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Screening for New Psychoactive Substances (NPS) without reference standards on High Resolution Mass Spectrometers (HR-MS)

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"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained."

Marie Curie

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- Maria von Cüpper -

RESUMO

As novas substâncias psicoativas, designadas por NSP, são consideradas um fenómeno mundial constituindo uma ameaça para a saúde pública. No término de 2017, mais de 670 NSP foram reportadas pelo Observatório Europeu da Droga e da Toxicodepência (*European Monitoring Centre for Drugs and Drug Addition*, EMCDDA).

O conceito NSP pode induzir em erro, dado que o termo "novas" pode levar à conclusão que se trata de substâncias recentemente introduzidas no mercado, contudo muitas delas foram desenvolvidas há mais de 40 anos por investigadores tendo em vista, normalmente, o desenvolvimento de novos fármacos. É o caso de John Huffman que pretendeu estudar as interações dos recetores canabinóides, de modo a testar os efeitos da canábis no cérebro humano. Estes compostos pretendem mimetizar os efeitos produzidos por substâncias psicoativas (cocaína, anfetaminas, *ecstasy*, entre outras), que se encontram sob controlo internacional, tendo um grande impacto nos seus consumidores. O que define as NSP? Segundo o Gabinete das Nações Unidas contra a Droga e o Crime (United Nations Office on Drugs and Crime, UNODC), estas são descritas como "novos estupefacientes ou substâncias psicotrópicas, na forma pura ou em preparação, que não são controladas pelas convenções das Nações Unidas referentes aos estupefacientes (1961) e psicotrópicos (1971), mas que podem representar um risco à saúde pública". As NSP são caracterizadas por pequenas modificações na estrutura química de substâncias (normalmente ilícitas), a substituição de apenas um átomo pode contribuir para um efeito mais poderoso, permitindo também contornar a legislação. Estas substâncias podem ser facilmente sintetizadas por qualquer pessoa que detenha conhecimentos em química e os laboratórios clandestinos onde a produção toma lugar situam-se predominantemente na China e em menor escala na Índia. A Internet é considerada o maior mercado de proliferação das NSP. Estas substâncias podem ser adquiridas nas chamadas "*smartshops*" como "*bath salts*" e "*research chemicals*" ou por "*street dealers*" que as obtêm em mercados online e depois são vendidas em discotecas, festivais de música e escolas, podendo ser designadas "*party pills*". É de salientar que, estes produtos são geralmente vendidos, contendo nos rótulos a descrição "não adequado para consumo humano", como uma estratégia de contornar as leis nacionais e internacionais. Os efeitos associados ao seu consumo, podem ser diversos e por vezes imprevisíveis. Podendo estes, variar entre efeitos estimulantes, alucinogénios e analgésicos. O consumo de NSP tem levado a intoxicações severas, bem como casos fatais.

O surgimento destes compostos, sob marcas e embalagens apelativas, estimula especialmente a curiosidade da geração mais jovem devido à sua acessibilidade e anonimato. De notar que, o ritmo acelerado com que as NSP aparecem nos mercados de drogas torna complicado o seu controlo. Quando uma substância chama a atenção das autoridades e, acaba finalmente por ser inserida na lista de substâncias controladas, rapidamente uma nova toma a sua posição, com o intuito de substituir aquela que foi banida do mercado.

A identificação destas substâncias revela-se desafiante para os analistas que estão dispostos em encontrar uma resposta eficaz para os casos que surgem, isto deve-se sobretudo às diferenças estruturais e à ausência de padrões de referência. De modo a enfrentar estas questões, um grupo de investigadores Dinamarqueses tiveram a ideia de desenvolver uma base de dados que compreendesse novas substâncias psicoativas como também compostos relacionados reportados por laboratórios de todo o mundo. Assim, o mesmo composto pode conter mais do que uma entrada, o que de certo modo contribui para a sua validação, podendo desta forma certificar-se que a informação fornecida é coerente. Atente-se que esta base de dados contempla elementos que são de cariz tanto experimental como não-experimental. Nesse sentido, esta tese consistiu em melhorar e testar esta biblioteca online universal (HighResNPS) destinada às NSP, quando os padrões de referência não se encontram disponíveis, utilizando a cromatografia líquida de ultra eficiência acoplada à espectrometria de tempo-de-voo (*ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry*,

UHPLC-QTOF-MS). O presente trabalho compreendeu duas partes fundamentais: a biblioteca e o método de *screening*. Relativamente à primeira, foi necessário assegurar que a informação apresentada no HighResNPS estava correta, em termos de iões fragmento, nomes IUPAC, classes de drogas, entre outros parâmetros. Graças à inclusão de duas *features* na base de dados, foi possível assinalar e corrigir 290 erros associados à massa do ião precursor e iões fragmento, encontrar a fórmula molecular em falta para alguns dos fragmentos e retificar 61 nomes IUPAC. É importante ter em consideração, que através do nome IUPAC pode ser gerado o InChIKey, que é um parâmetro de relevância máxima, visto que é característico de um único composto, permitindo facilmente a sua identificação. Além disso, pode ser útil para proceder a uma pesquisa na Internet, isto é, noutras bases de dados online. Note-se, que as substâncias podem apresentar diferentes designações, do mesmo modo, os nomes IUPAC também podem sofrer ligeiras variações, pelo que o InChIKey é crucial para reconhecer a identidade do composto em questão. Verificou-se que nem sempre, foi possível, encontrar o nome IUPAC e consequentemente o InChIKey, o que pode ser crítico especialmente quando isómeros posicionais estão presentes.

O constante aparecimento de duas classes específicas de NSP requereu uma especial atenção. Duas subbibliotecas foram criadas, a primeira contendo 51 análogos do fentanil e a segunda, 215 canabinóides sintéticos. Por forma, a que fornecessem uma visão geral quanto aos padrões de fragmentação, sendo estes posteriormente comparados com os resultados presentes na base de dados. Em geral, o estudo da fragmentação para ambos os grupos de compostos, revelou que novos derivados podem ser previstos, tendo em conta, os princípios estabelecidos. No que diz respeito à segunda parte, a aplicabilidade do método de *screening* foi avaliada tendo como base o HighResNPS. Durante este processo, foi explorada uma nova perspetiva, em como usar o HighResNPS sem necessidade de o importar. Assim, foi utilizada como uma base de dados espectral online, possibilitando o acesso imediato às informações mais recentes. De modo a testar o HighResNPS, 36 amostras confiscadas pelas autoridades Dinamarquesas durante o período de 2015 e 2019 foram analisadas utilizando UHPLC-QTOF-MS. Contudo, apenas 14 amostras de NSP desconhecidas foram selecionadas, uma vez que as restantes deram origem a resultados idênticos ou indicaram a presença de drogas tradicionais, tal como heroína e MDMA.

No âmbito deste trabalho, a ionização por *electrospray*, no modo positivo, foi utilizada como fonte de iões. A deteção foi realizada com base numa aquisição de alta resolução de massa exata, através de um espectrómetro de massa de tempo-de-voo. Todos os dados foram processados com recurso ao *UNIFI 1.9.2* e adquiridos por meio de um varrimento completo, com base numa aquisição independente de dados (AID) (*data independent acquisition*, DIA). O processo de fragmentação foi induzido por uma rampa de energia de 10 a 40 eV. Para todas as 14 amostras, o ião precursor foi investigado no HighResNPS, utilizando pelo menos uma décima, de modo a reduzir, assim o número de potenciais candidatos, tendo como base a massa exata do composto. De seguida, o perfil de fragmentação foi examinado, correlacionando os dados obtidos com a informação disponível na biblioteca. Em suma, esta abordagem permite obter uma informação preliminar, podendo conduzir-nos frequentemente à NSP certa. Contudo, em alguns casos nem sempre é possível definir uma estrutura química rigorosa do composto, especialmente quando estamos perante isómeros posicionais. No entanto, podemos ter uma noção clara da classe de drogas a que este deve pertencer. Sendo, assim necessário nestas situações recorrer a outras metodologias como por exemplo, espectroscopia de ressonância magnética nuclear (RMN) (nuclear magnetic ressonance, NMR) ou adquir os padrões de referência, de modo a conseguir um resultado o mais exato possível.

Palavras-chave: NSP; *HighResNPS*; *screening;* AID.

ABSTRACT

New Psychoactive Substances, commonly referred to as NPS, are continuously emerging on the recreational drug market under appealing trade names and packages, sparking especially the curiosity of the youngest generation. These compounds are designed to mimic the effects of traditional illicit drugs and characterised by slightly modifications in their chemical structures. In this way, NPS represent a global concern to the public health owing to their unpredictable pharmacological and toxicological effects, thus leading to severe intoxications that might culminate in deaths.

The term "new" could mislead their real definition, since some of them have been manufactured more than 40 years ago for distinct purposes and recently, they have been reintroduced on the drug scene thanks to their psychoactive properties. In addition, they hold a label "not for human consumption", in order to divert the attention from their actual composition and therefore, avoiding national and international laws. The identification of NPS poses a challenge for the analysts that are willing to give an effective answer to the cases at hand, this is mainly due to structure similarity and lack of reference standards. In this way, this thesis aimed at improving and testing a universal online library named HighResNPS for NPS, when reference standards are not readily available, using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Hence, the presented work was composed by two main parts: the database and the screening method. It was essential to ensure that the information contained on the HighResNPS was correct, in terms of fragment ions, IUPAC names, drug classes, among other parameters; in addition, the steady emergence of two specific NPS drug classes forced us to create two additional sub-libraries containing 51 fentanyl analogues and 215 synthetic cannabinoids that could provide an insight into their fragmentation patterns, which were further compared with the data available on the database. In general, the fragmentation study for both groups revealed to be successful and therefore, novel derivatives can be predicted based on the established principles.

Regarding the second part, the applicability of a screening method for NPS in seizures was evaluated using HighResNPS as an online mass spectral database. This analysis was performed exclusively to 14 seizures that were confiscated by the Danish authorities between 2015 and 2019 containing unknown compounds. Furthermore, the mass spectral was acquired in positive mode and in data-independent acquisition (DIA), being the fragmentation process induced by a ramp of collision energy (CE) from 10 to 40 eV. For all the aforementioned samples, the precursor ion was searched on HighResNPS by selecting at least one decimal, in order to reduce the number of potential candidates based on the accurate mass and then, the fragmentation profile was examined, correlating the data obtained with the one available on the database. In conclusion, the approach provides a preliminary information and it might often lead us to the correct NPS. However, in some cases it is not always possible to define the exact structure of the compound, nevertheless we can have an idea concerning the drug class within it might be included, this is observed for positional isomers when it is required to resort to other methodologies such as, nuclear magnetic resonance (NMR) spectroscopy or purchase the reference standards to obtain an unambiguous result.

Keywords: NPS; HighResNPS; screening; DIA.

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ABBREVIATIONS

% v/v	Volume per volume
ºC	Degrees Celsius
4-MMC	Mephedrone
ACMD	Advisory Council on the Misuse of Drugs
AIs	Aminoindanes
AM	Alexandros Makriyannis
AU	Australia
BZP	1-Benzylpiperazine
CA	Canada
CAT	Methcathinone
CB1/CB2	Cannabinoid receptor type 1/ type 2
CE	Collision energy
CID	Collision-induced dissociation
CNS	Central nervous system
CP	Charles Pfizer
Da	Dalton
DAT	Dopamine transporter
DDA	Data dependent acquisition
DE	Germany
DIA	Data independent acquisition
DK	Denmark
DOB	2,5-Dimethoxy-4-bromoamphetamine
DOC	2,5-Dimethoxy-4-chloroamphetamine
DOI	2,5-Dimethoxy-4-iodoamphetamine
e.g.	*exempli gratia*
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ES	Spain
ESI	Electrospray ionization
EU	European Union
Europol	European Union Agency for law enforcement cooperation
eV	Electron volt
EWS	Early Warning System
F1mass/ F2mass /F3mass	Mass of the three most intense fragments
F1MF/ F2MF/ F3MF	Molecular formula of the three most intense fragments
FI	Finland
GC-MS	Gas chromatography-mass spectrometry
GR	Greece
h	Hour
HRMS	High resolution mass spectrometry

HSS	Hollow structure section
HU	Hebrew University
i.e.	*id est*
InChIKey	International Chemical Identified Key
IT	Italy
IUPAC	International Union of Pure and Applied Chemistry
JWH	John W. Huffman
L/h	Litre per hour
LC-MS	Liquid chromatography-mass spectrometry
LGBT	Lesbian, gay, bisexual and transgender
m/z	Mass-to-charge ratio
MDMA	3,4-Methylenedioxymethamphetamine (ecstasy)
mg	Milligram
min	Minute
mL	Mililiter
mM	Milimolar
mm	Milimetre
MS	Mass spectrometry
MS^E	Type of broadband data-independent acquisition mode
MXE	Methoxetamine
NAT	Noradrenaline transporter
NMR	Nuclear magnetic resonance
NO	Norway
NPS	New Psychoactive Substances
PCP	Phencyclidine
PEA	Phenethylamines
QTOF	Quadrupole time-of-flight
rpm	Revolutions per minute
R_t	Retention time
SCs	Synthetic cannabinoids
SERT	Serotonin transporter
TFMPP	3-Trifluoromethylphenylpiperazine
THC	Delta-9-tetrahydrocannabinol
TIC	Total ion chromatogram
TMCP	2,2,3,3-Tetramethylcyclopropyl
UHPLC-QTOF-MS	Ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry
UK	United Kingdom
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
V	Volt
µg	Microgram
µL	Microliter

1 INTRODUCTION

In this chapter, a literature review is presented comprising the relevant topics regarding the conducted work. The control of NPS in Europe, their classification system, the use of a database (HighResNPS) and the analytical tools applied for the screening of these drugs in seized material are described.

1.1 Into the world of NPS

New Psychoactive Substances, the so-called NPS, are a worldwide phenomenon that poses a threat to the public health [1]. In the end of 2017, more than 670 NPS were recorded by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [2].

The NPS concept might mislead its real definition, *i.e.* the term "new" could lead naturally to the conclusion that these substances are novel, however some of them were synthesized more than 40 years ago by researchers (*e.g.* John W. Huffman) in an attempt to improve the medical properties of cannabis [3]. More recently, their recreational use came into play [4]. The main purpose of these drugs is to mimic the effects generated by well-known substances (*e.g.* cocaine, ecstasy, amphetamines and fentanyls [5]), which are under control, producing a stronger impact on their users [1]. Hereupon, what define NPS? According to the United Nations Office on Drugs and Crime (UNODC), they are described as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat" [6]. In other words, NPS also referred to as designer drugs, are characterized by a modification in their chemical structure; sometimes a replacement of a hydrogen for a halogen is enough to contribute to a more powerful effect and consequently, to circumvent the legislation [7]. These substances can be easily manufactured by people who have an expertise in chemistry and the clandestine laboratories where their synthesis takes place, are predominantly based in China and less frequent in India [1]. Afterwards, the drugs are distributed through the borders, maritime, air and terrestrial [8]; until reach their destination, which is mainly Europe or America [1]. Thus, the proliferation of NPS is evident all over the Internet [9], the major marketplace, but not only; they can also be purchased on smartshops as "bath salts" and "research chemicals" or by "street dealers" who obtain them from online shopping and then sell them in clubs, at music festivals and schools [1]. In this way, they could be described as "party pills" [10]. In addition, the products on sale hold a label "not for human consumption" as a strategy, in order to avoid national and international laws [11]. The effects associated to its intake, may be diverse and sometimes unpredictable, but might range between stimulant, hallucinogenic and analgesic side effects, just to name a few [12].

The rising wave of "legal highs" [13] has sparked the curiosity among the youngest generation specially owing to their affordability and anonymity [1]. Besides that, the trade names given, and the packages used to sell NPS can also be attractive factors that make people want to try them out [8]. In general, males are the most common consumers of these substances [6,14]. Recently, a study carried out in six European countries (Ireland, Germany, Hungary, the Netherlands, Poland and Portugal) reported that males between 18 and 24 years old are in fact, the typical NPS users and depending on their living place (big or small cities) as well as, education level, they can be categorized into three groups (marginalized, nightlife and online users) [6]. Therefore, the population target includes not just students and clubbers, but also LGBT, psychonauts, homeless and prison communities [15,16].

The fast-pace at which NPS emerge on the drug markets, makes it challenging to control them. When a drug comes to the attention of the authorities and finally is added on the list of controlled substances, a new one appears with the aim to replace it [17]. This situation is rising frustration for the policy makers who are

willing to find an effective approach to regulate NPS [18]. So, why is this so complex? The problem is that these drugs are recent on the market and therefore, their characterization is still under investigation once there is scarce analytical information on NPS and lack of reference standards [8]. In this way, how can we detect NPS in seized material? This thesis aims at building a universal NPS screening library that can be employed by all high resolution mass spectrometers (HR-MS) without the use of reference standards.

1.2 The control of NPS in Europe

The rapid growth of NPS on the European drug market forced the authorities to find a reasonable method to tackle this issue and consequently, to protect the health of EU citizens [19]. Thus, the EMCDDA in collaboration with the European Union Agency for law enforcement cooperation (Europol) implemented in 1997 the European Union Early Warning System termed EWS, which is responsible for monitoring and detecting these novel drugs. In addition, this advisory system comprises 30 national early warning systems (28 member states, Norway and Turkey) and law enforcement networks such as, the European Medicine Agency and the European Commission providing risk assessments on NPS and communicating the information collected to the member states, so they can define their own preventive actions [20].

In Europe, the NPS legislation system consists of a three-step process: 1) early warning or information exchange, 2) risk assessment and 3) control measures. The EMCDDA oversees the first two stages, which includes the management of the EWS and the risk assessments, while the last action is ruled by the European Commission, the Council of the European Union and the European Parliament [21]. This approach was strengthened in November 2017 with the purpose to improve the Union's response to NPS [22]. So, how does it work? Considering the analysis of seized material, if any compound is suspected to be a NPS, the national early warning system should announce immediately the EMCDDA and provide chemical and analytical information, besides that the circumstances of the event may also be investigated. In case the suspicion is right, a formal notification is issued by the respective country [23]. This comprises the following relevant aspects: the name and identifiers of the substance, the properties (chemical and physical), as well as the analytical methodologies for its identification, the pharmacology and toxicology [24]. At this point, the monitoring of the substance takes place. In this way, the member states are aware about the new drug and therefore, ready to act accordingly. For example, in forensic laboratories it might be essential to include the substance in their analytical screening, particularly when the recent information can be helpful in analysing data that might have been missed during previous studies owing to the lack of knowledge [20]. The first stage is completed after the evaluation of the need of a formal risk assessment, which means that the information regarding the novel substance is investigated and it will be documented on the so-called Joint Report (initial report). In this way, the Council may decide whether to undertake a risk assessment of the substance or not [24]. It is also during this process that the potential risks and implications of adding it on the controlled substances list are examined. Furthermore, this drug is further subjected to a comparison with the compounds under control as well as others containing similar features. However, when the substance reveals therapeutic benefits or it is applied in industry for developing medical products there is no need to conduct a risk assessment [24]. This step is complete after the formulation of a report by the Scientific Committee. It should encompass an analysis description and any opinions regarding the compound, more specifically: chemical and physical characteristics of the NPS in consideration, as well as the mechanisms of action, which may also include the medical value; potential risks for health and society; information concerning any detection by the authorities and its manufacture; definition of control measures in the member states and the chemical precursors used for its fabrication [25]. Lastly but not least, the introduction of control measures which is decided by the Council and represents the final step. At this level, the member states need to define the rules and criminal penalties, they have one year from the date of the

decision to act in accordance with their national law [24]. In this way, what is the criteria to add a certain compound under control? Firstly, it is important to make sure that its psychoactive properties are clear, as well as the probability of generating abuse or dependence, which may result in hallucinations or disturbances in behaviour or perception, and the impact on public health. Furthermore, its use in medical therapy should be also taken into account [26].

1.3 NPS categories

The UNODC classifies NPS into six main categories: **synthetic cannabinoids**, **synthetic cathinones**, **ketamines**, **phenethylamines**, **piperazines** and **plant-based substances**. Besides these classes, a seventh category can be mentioned – the miscellaneous, which corresponds to substances that do not fall into any of the indicated groups, such as aminoindanes, phencyclidine-type substances, tryptamines and benzodiazepines [27]. This classification system is based on the chemical structure which characterizes the different compounds and allows the development of analytical methods for a proper identification [28]. A general description for each category is presented below.

1.3.1 Synthetic cannabinoids

According to the EMCDDA, synthetic cannabinoids are widely controlled in Europe [29]. This class of compounds also referred to as SCs [30], emerged in the early 1960s with the purpose of boosting the anti-inflammatory and analgesic properties of delta-9-tetrahydrocannabinol (THC)[a] [31,32], which is the main psychoactive component in the plant *Cannabis sativa*, also known as marijuana [33]. Along the years, cannabis has been documented thanks to its medical benefits (*e.g.* 500 years ago the extracts of marijuana were applied to alleviate cramps and to treat malaria in ancient China) [30]. However, more recently its recreational use has restrained the initial purpose [33]. Thus, it is considered a narcotic drug and is forbidden by the United Nations Single Convention on Narcotic Drugs mainly due to its uncertain pharmacological effects and psychoactive properties [30].

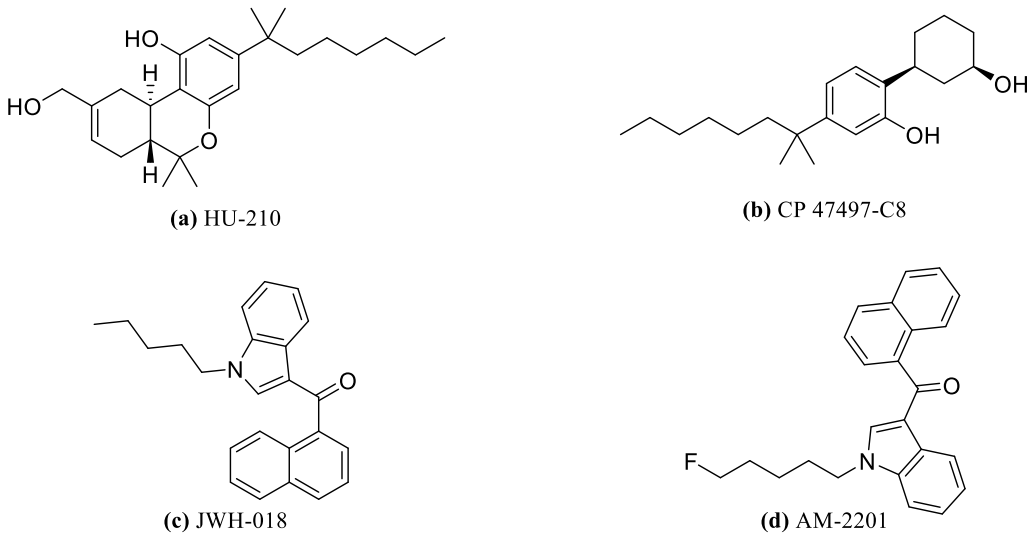
SCs bind to cannabinoid receptors of the organism, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) generating effects, which are like the ones occurring in THC *e.g.* relaxation, disinhibition, euphoria and distorted perception [34]. These receptors can be found in different parts of the body, CB1 is mostly present in the central nervous system whereas CB2 is predominantly located in the cells of the immune system [35]. Although SCs and THC stimulate the same receptors, it is reported that the consume of SCs leads to a great number of hospital entrances as well as, a higher level of toxicity [31]. This can be explained in terms of affinity [32], *i.e.* the measure of how strongly a drug molecule binds to a receptor [36]. In light of above, the cannabinoid receptors have a higher affinity for SCs, which are recognized as full agonists [32], this means that SCs bind to receptors producing a full response [36], while THC is a partial agonist [32], in other words it acts as an agonist however, the effect generated is not as great [36], thus being less potent [37].

It is worth noting that SCs are synthesised in laboratories (as powders) and not coming from Cannabis, which is sometimes misunderstood [37]. Instead, most of these compounds are chemically fabricated in China and shipped to Europe, where their aspect is modified with the use of herb mixtures and solvents *e.g.* acetone or methanol, in order to disguise their real composition and therefore, make people think that the product is provided by natural sources [32]. As a result, the consumers should suspect of the content, since unknown chemical compounds can be added and in different compositions, which may lead to unpredictable effects [37].

[a] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

In the 2000s, new variations of SCs started to arise on Internet drug markets. Most of these novel compounds are commercialized under the brand names such as "Spice", "K2" or "Kronic", depending on the part of the globe where they are sold. The first designation is more common in Europe; the second in the USA and the last one in Australia and New Zealand [32]. They are usually on sale as legal highs and with a label describing that they are not for human consumption; additionally, no information regarding the health risks is mentioned [32]. However, the drug users' who are mainly young people [38], report their preference for SCs specially because they can obtain these substances easily on Internet websites and smartshops on the condition of anonymity at a low price and they are a legal alternative to marijuana; besides that, the lack of reference standards make difficult their detection during a drug testing which is therefore limited to THC [32]. Concerning the way of administration, they can be rolled in a paper cigarette, inhaled through an electronic cigarette or taken as an herbal infusion [32]. It is important to highlight that the consume of SCs is strongly associated with acute intoxications and sometimes overdose deaths may take place [39]. However, other side effects may occur but not as severe as aforementioned such as, tachycardia, chest pain, lethargy, nausea, hallucinations, amnesia, paranoia, panic attacks, shaking and renal damage [32,34].

In 2010, an array of SCs started to be international banned [32]. Nevertheless, the efforts were not enough to control the rapid emergence of these compounds, since every year a diversity of structures is launched on Internet drug markets [32]. In this way, it is challenging to regulate this type of substances [32]. The drugs available on online shopping and smartshops are homologues of SCs, which were first manufactured in the 1960s by researchers from different institutions or companies and to distinguish them, they have associated a code name derived from their initials [32]. "HU" series comes from the Hebrew University in Jerusalem, "CP" corresponds to Charles Pfizer & Company, "JWH" belongs to John W. Huffman and "AM" to Alexandros Makriyannis just to name a few [40]. In **Figure 1.1**, some examples of the structures are given: HU-210 **(a)**, CP 47497-C8 **(b),** JWH-018[b] **(c)** and AM-2201[b] **(d)**, respectively [30].



(a) HU-210 **(b)** CP 47497-C8 **(c)** JWH-018 **(d)** AM-2201

Figure 1.1 | Examples of the main SCs series.

More recently, the EMCDDA stablished a method for naming compounds containing a long chemical name that can also be applied to emerging novel substances on the black market [40]. In order to simplify, the code names are based on the International Union of Pure and Applied Chemistry (IUPAC) name of SCs and are given accordingly to the initials representing the structures present in the compound [40]. For example, APICA

[b] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

illustrated in **Figure 1.2**, has the following systematic name *N*-(1-adamantyl)-1-pentyl-1H-indole-3-carboxamide which confirms the criteria *i.e.* A stands for adamantyl, P denotes pentyl, I designates indole and CA represents carboxamide. Furthermore, SCs can be characterized by four units: core, tail, linker and linked group [40].

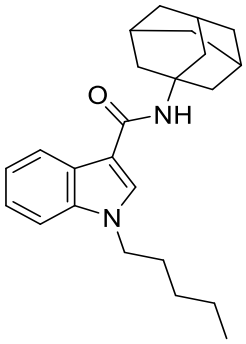


Figure 1.2 | APICA.

The Advisory Council on the Misuse of Drugs (ACMD) reported that SCs can be classified into the main sub-groups: naphthoylindoles, naphthylmethylindoles, naphthoylpyrroles, phenylacetylindoles, cyclohexylphenols and classical cannabinoids **(Figure 1.3)** [30,41-43]. These categories have been in use until recently, however the rapid growth of SCs contributed to an outdated classification system. Since then, a number of chemical modifications have been stated, for example: the indole ring, which is common to JWH compounds was replaced by an indazole ring; furthermore, the carboxamide group and adamantane or quinoline ring make now part of their structure [44].

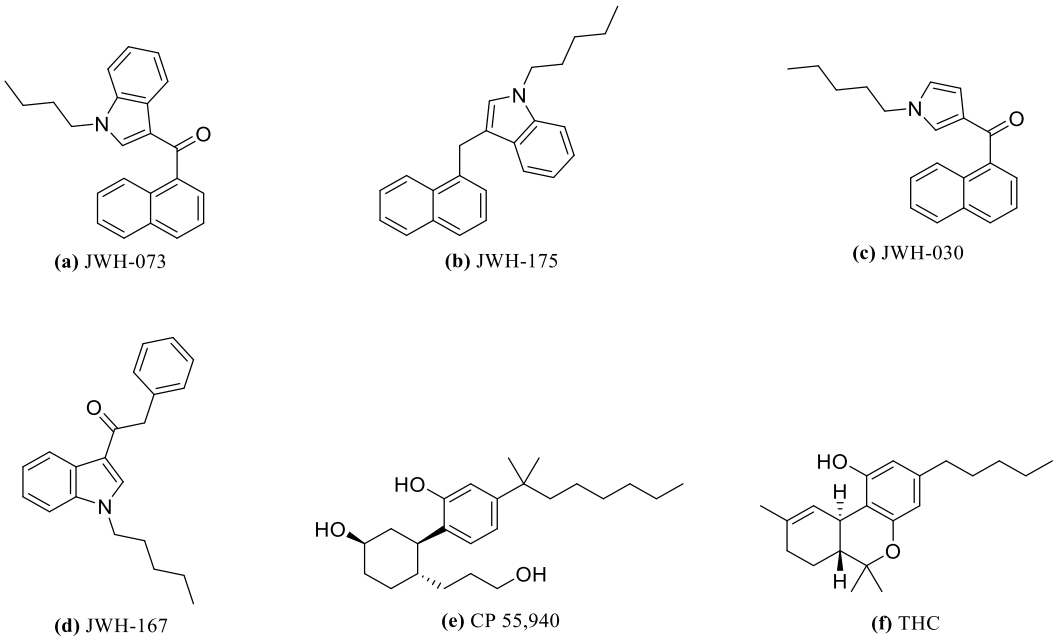


Figure 1.3 | Examples of compounds belonging to the main SCs sub-groups: (a) naphthoylindoles, (b) naphthylmethylindoles, (c) naphthoylpyrroles, (d) phenylacetylindoles, (e) cyclohexylphenols and (f) classical cannabinoids.

1.3.2 Synthetic cathinones

Synthetic cathinones are recognized as the second predominant group of NPS monitored in Europe right after SCs [45]. This class of drugs consists of chemical analogues of natural cathinone **(Figure 1.4a)**, which is a

psychostimulant alkaloid present in the leaves of a plant named *Catha edulis* also known as khat, mainly found in northeast Africa and Arabia Peninsula [46]. In addition, natural cathinone produces amphetamine-like effects; the relationship between this substance and amphetamine is illustrated in **Figure 1.4**.

Figure 1.4 | (a) Natural cathinone and (b) amphetamine.

Thanks to their ability to generate stimulant effects on the central nervous system (CNS), cathinones were originally applied for medical purposes, especially to treat patients with Parkinsonism [47]. However, in the past few years their recreational use became relevant with the first appearance of two compounds, methcathinone (CAT) **(Figure 1.5a)** and mephedrone (4-MMC) **(Figure 1.5b)** [48]. Nevertheless, the psychoactive properties of these derivatives were soon identified and consequently, their consume was forbidden [47]. In this way, modifications in the structure of these drugs took place and hence, novel analogues started to emerge [49].

Figure 1.5 | (a) Methcathinone and (b) mephedrone.

In light of above, synthetic cathinones exert their action on CNS by inhibiting monoamine transporters such as, dopamine transporter (DAT), noradrenaline transporter (NAT) and serotonin transporter (SERT) [47,50]. The affinity to these transporters might be different from derivative to derivative due primarily to variations in its chemical structure. Besides that, it should also be taken into account that the age, health condition, use of medication, intake of other substances of abuse and alcohol could influence the length of its action [47].

These novel drugs commonly referred to as "bath salts" are sold under attractive brand names: "White Lightning", "Bloom", "Vanilla Sky", among others [51]. They can be found in the form of a powder, brown or white crystal-like, inside a plastic package holding a label "not for human consumption", although "jewellery cleaner" and "phone screen cleaner" are also typical designations [51,52]. In addition, it is important to reinforce that these packages might contain not only one compound, but instead a mixture of substances - caffeine and other illegal drugs - can be identified [53].

It is worth noting that these drugs are intended for users that seek for legal substitutes of illicit psychostimulants such as, cocaine, methamphetamine and MDMA ("ecstasy") [54]. Concerning the way of administration, they can be orally, intravenously and intranasal administered [13]. Consequently, serious side effects to the human health can arise no matter the exposure degree: panic, hallucinations, paranoia, aggression and violence (*i.e.* suicide and homicide might be committed), chest pain, nausea, tachycardia and therefore, could culminate into death due mainly to liver and kidney failure [54,55]. The latter reactions could last approximately 3 to 4 hours [56]. Over the course of the years, several fatalities have been linked to the consumption of these drugs, especially mephedrone, methylone and pentylone [2].

These legal highs came originally from natural cathinone and are considered chemical analogues of methcathinone [57]. Based on their generic chemical structure **(Figure 1.6)**, synthetic cathinones are characterized by a carbonyl group attached at the beta position on the amino alkyl chain, thus they are usually described as "bk-amphetamines" [58].

A diversity of novel chemical entities can be easily generated by including new functional groups to the core skeleton, replacing one or more substituents on the phenyl ring (R_1 and R_2), at the alpha-carbon position (R_3) and at the *N*-alkyl chain (R_4 and R_5) [59].

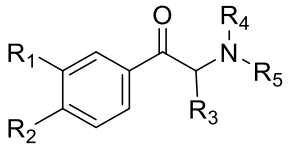


Figure 1.6 | General structure of synthetic cathinones.

Currently, the cathinone derivatives can be divided into the four groups as depicted in **Figure 1.7** [47,49]:

1. Consists of *N*-alkyl compounds or alkyl and halogen substituents in any position of the aromatic ring (*e.g.* flephedrone and 4-chloroethcathinone);
2. Considers compounds with methylenedioxy groups in any position of the phenyl ring (*e.g.* methylone[c] and pentylone);
3. Includes analogues of natural cathinone with a pyrrolidinyl substitution at the nitrogen atom (*e.g.* MPHP and 4-fluoro-PV9);
4. Contains the methylenedioxy and *N*-pyrrolidinyl substituents (*e.g.* MDPPP and 3,4-MDPV[c]).

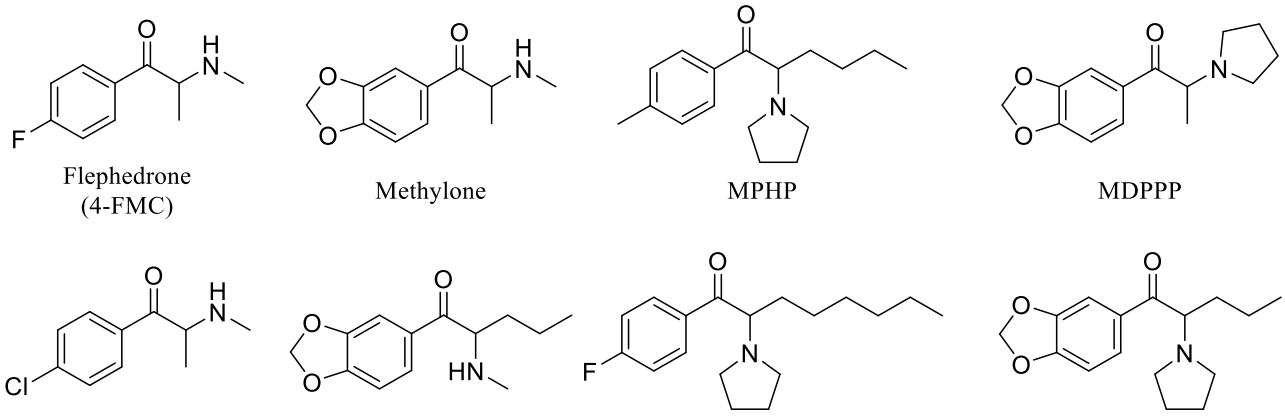


Figure 1.7 | Examples of cathinone derivatives for each group.

[c] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

1.3.3 Ketamines

The scientist responsible for the first appearance of ketamine **(Figure 1.8a)** was Calvin Stevens from the Parke-Davis Pharmaceutical Company (Michigan, USA), which synthesized this compound in 1962 with the aim to discover an alternative to the hallucinogenic agent phencyclidine (PCP) **(Figure 1.8b)** [60]. Thanks to its anaesthetic properties and rapid onset action, ketamine was primarily applied in the clinical field *e.g.* to treat problems with alcohol, heroin addiction, anorexia and depression [60,61]. However, it was later found that this substance would be linked to psychedelic episodes, affecting the perception and mental state of patients [61]. In this way, its use has been restrained [62].

(a) (b)

Figure 1.8 | (a) Ketamine and (b) phencyclidine (PCP).

In the 1970s, this group of compounds emerged on the illegal drug market [62]. At first, the users were healthcare professionals who could easily have contact with the drugs, but this scenario has changed a few years later and ketamines started to be a trend among club goers; in other words, they were commonly seen on the "rave scene" as an adulterant to ecstasy pills [62,63]. The effects induced by its intake rely on the dose [61]:

- speech disorder, disorientation, anxiety and irrational behaviour (low doses);
- respiratory disturbances, complications in moving, seizures and nausea (high doses);

Lastly, loss of consciousness and paranormal experiences may occur at excessive dosage that may not exceed 2 mg/kg [61]. Thus, ketamine is sometimes referred to as "dissociative drug" [64] due to the sense of disconnection of the mind from the body experienced by its users [65]. However, overdose deaths are unlikely to take place owing to its broad therapeutic window [65] .

These drugs of misuse are available on the black market as white powders and tablets [61] and are commercialized under appealing street names such as, "Special K", "Vitamin K", and "Kit Kat" [66]. Likewise in other NPS, ketamines are usually adulterated or combined with other components *e.g.* PCP, MDMA, cocaine, diazepam and paracetamol [61]. Concerning the administration routes, they can be intravenously, orally and intranasal administered [65].

Ketamine belongs to the arylcyclohexylamines group [67]. This class of compounds consists of a cyclohexylamine unit with an aryl moiety (*e.g.* phenyl ring) attached to the atom to which the amine group is also linked [67].

The urge to manufacture novel analogues became relevant when ketamine started to be controlled in different countries [64,68]. In this way, methoxetamine (MXE) **(Figure 1.9)** was one of the outstanding substances produced. This compound differs from ketamine in the following aspects [63]:

- the inclusion of the *N*-ethyl group that could boost the potency as well as its duration of action;
- the replacement of the 2-chloro with the 3-methoxy group which could suggest the mitigation of its anaesthetic and analgesic features;

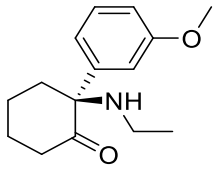


Figure 1.9 | Methoxetamine.

However, MXE was later banned from the drug market [62]. Hence, new analogues were fabricated **(Figure 1.10)** [62].

(a) (b)

Figure 1.10 | Examples of new ketamine analogues: (a) 2-methoxyketamine and (b) *N*-ethylnorketamine (*N*-EK).

1.3.4 Phenethylamines

Phenethylamines (PEA) [69] are famous for their hallucinogenic properties [70]. Mescaline[d] **(Figure 1.11)** is the first alkaloid reporting these effects, which are produced by the peyote cactus (*Lophophora williamsii*) [71]. In order to achieve a full reaction, it is recommended a high dose between 250-500 mg, which means that its effectiveness is poor. Nevertheless, this substance is believed to be the foundation to which new molecules are designed [71].

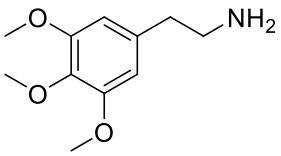


Figure 1.11 | Mescaline.

It is worth noting that this class of compounds comprises amphetamine **(see Figure 1.4b)**, methamphetamine **(Figure 1.12a)** and MDMA **(Figure 1.12b)**, which are controlled under the 1971 Convention on Psychotropic Substances [72], therefore not considered NPS. They are usually found in the form of powders and tablets, being generally ingested [27]. In addition, they are defined by different street names such as, "RDJ" and "Europa" [27].

(a) (b)

Figure 1.12 | (a) Methamphetamine and (b) MDMA.

In terms of side effects, PEA are responsible for hallucinogenic and stimulant responses in the CNS, which can lead to disorientation, vertigo, nausea, headaches, among other symptoms [73]. Concerning the fatalities record, the cases of heart attack stated are not many [74].

[d] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

PEA are part of a major family of NPS named arylalkylamines [75] that encompass some of the following sub-groups: "2C series", "D series", NBOMe and benzodifurans [72]. A brief description for each one is presented below.

"2C series" (Figure 1.13) demonstrate a certain resemblance to the chemical structure of MDMA and it is suggested that they correspond to analogues of mescaline [72]. Alexander Shulgin was the organic chemist responsible to give this designation to these substances, in order to represent the two carbons between the benzene ring and amino group **(see Figure 1.11)** [70]. Apart from that, he was the author of a book entitled "PIHKAL: A Chemical Love Story", which comprises the information concerning the effects and dosages along with guidelines for the synthesis of more than 200 hallucinogenic compounds [72].

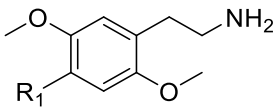


Figure 1.13 | General structure of 2C series. R_1 represents the possible substituents [72].

In 1991, its publication led to a rise in popularity of the substances documented (*e.g.* 2C-B, 2C-T-7, 2C-E, 2C-D, 2C-C, 2C-I, 2C-T-2, 2C-T-4, 2C-H, 2C-N and 2C-P) and consecutively to their restriction [72]. This subgroup can be characterized by a methoxy group at the positions 2 and 5 on the aromatic ring and a substituent (*e.g.* a halogen, alkyl and alkylthio moiety) at the para-position, which might produce an unlimited number of derivatives **(Figure 1.14)** [70]. The potency of each substance may vary according to the substitution at the position 4 on the aromatic ring [72]. It is reported that derivatives containing halogen substitutes are more powerful (*i.e.* the hallucinogenic effects are stronger) than the ones without these electronegative elements [72].

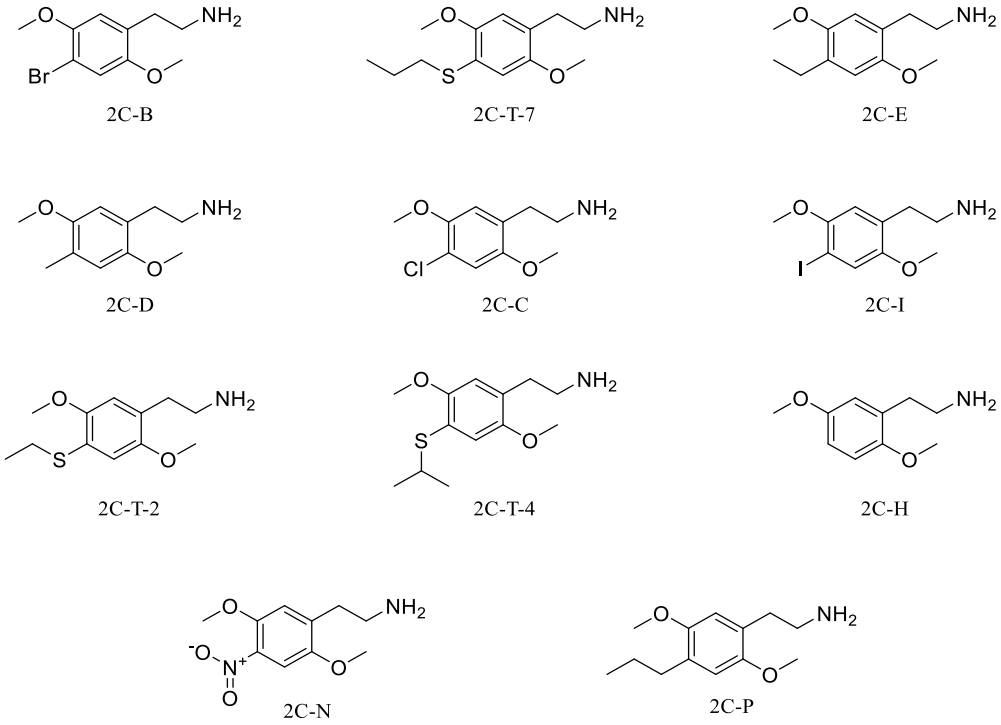


Figure 1.14 | Examples of 2C series compounds from the book "PIHKAL: A Chemical Love Story" [72].

From the chemical point of view, **"D series" (Figure 1.15)** involve the inclusion of a methyl group on the alpha carbon of the side chain, which differs slightly from the "2C series" [27].

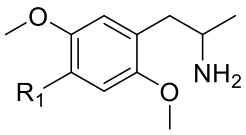
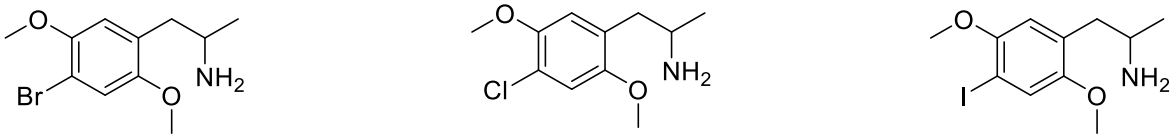


Figure 1.15 | General structure of D series. R_1 represents the possible substituents.

DOB[e], DOC and DOI are some examples that make part of this sub-group **(Figure 1.16)** [27].



DOB DOC DOI

Figure 1.16 | Examples of D series.

Afterwards, some adjustments were made on the "2C series", therefore resulting in novel analogues [72]. These new compounds correspond to the **2C-X-fly series**, to which X refers to any substitute at the position 8 and fly is attributed to two dihydrofuran rings attached to both opposite sides of the benzene ring **(Figure 1.17a)** [72]. Further, the aromatization of these heterocyclic compounds produced a different series named **"dragonfly"**; this term suggests that the chemical structures can be compared to an insect **(Figure 1.17b)** [72]. Besides that, this group of drugs are reported to be extremely potent [72].

(a) 2C-B-fly **(b)** Bromo-Dragonfly

Figure 1.17 | The benzodifurans sub-group – an example for each series: (a) 2C-X-fly and (b) "Dragonfly" [76].

In 2011, the **25-X-NBOMe** derivatives of the "2C series" emerged on the recreational market [12]. These series are of major concern [70], since their intake can result in hallucination and delirium, which can be felt in a greater degree than in other 2C compounds [77]. In addition, they are associated to repeated serious intoxications and deaths may occur [77]. The NBOMe series consists of methoxy groups at the positions 2 and 5 on the benzene ring and replacement of one hydrogen (R_3) from the amino group by an *N*-2-methoxybenzyl moiety **(Figure 1.18a)** [77]. However, further structural modifications were performed [77]. For example, 2,3-methylenedioxy or hydroxy groups were used in placed of the methoxy group from the *N*-2-methoxybenzyl moiety [77].

[e] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

Moreover, 2,5-dimethoxybenzene is replaced by benzofurane (5-APB-NBOMe) **(Figure 1.18b)** or 3,4,5-trimethoxybenzene (mescaline-NBOMe) **(Figure 1.18c)** giving rise to novel representatives [77].

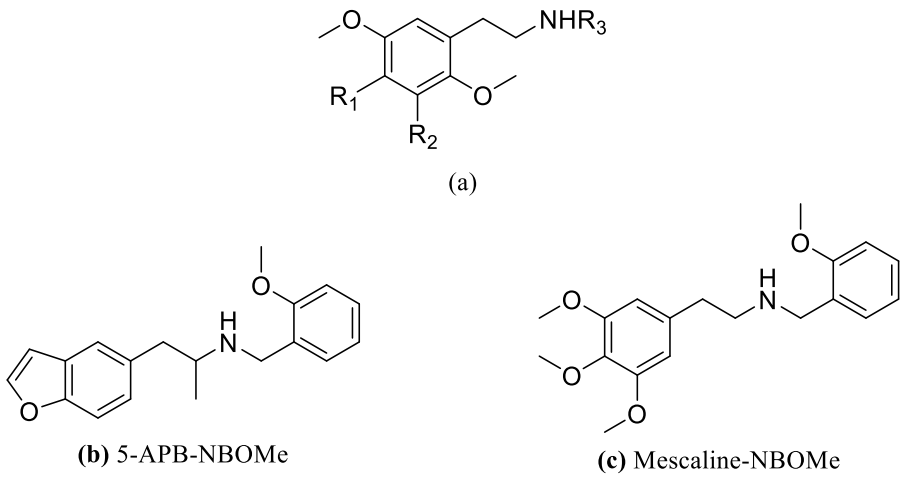


Figure 1.18 | (a) General structure of NBOMe series where R_1, R_2 and R_3 are the substituents, (b) and (c) that correspond to novel representatives [77].

1.3.5 Piperazines

At first, these compounds were developed for therapeutic purposes but rapidly their psychoactive properties came into play [27]. In the late 1990s, this class of substances appeared on the drug market [78] with the aim to mimic the stimulant effects of MDMA; however, its potency is inferior [12]. In this way, piperazines derivatives can be generally found in ecstasy tablets [79]. Currently, these drugs are not included in any of the United Nations Conventions (1961 and 1971) [5,80], except 1-benzylpiperazine (BZP) **(Figure 1.19a)** which is already under international control [80].

Figure 1.19 | (a) BZP and (b) TFMPP [80,82].

These drugs are available in tablets or powders [81] and they can be consumed alone or as a blend [74] (*i.e.* a combination with other substances, such as a second piperazine, caffeine, cocaine and MDMA) [82], which may be taken orally or by snorting [81]. In addition, they are usually found under some of the following trade names: "Bliss", "Rapture", "Legal X" [83]. In case of BZP, this chemical is commonly mixed with 3-trifluoromethylphenylpiperazine (TFMPP) **(Figure 1.19b)**, another piperazine derivative, to promote a long-term reaction, usually up to 8 hours [82]. Concerning the side effects, they may depend on the dosage; high portions could lead to hallucinations, disorientation, headaches and difficulties in breathing. In addition, seizures, kidney failure and fatal intoxications are also reported [84].

Piperazines **(Figure 1.20a)** consist of a six-membered ring containing two nitrogen atoms in opposite positions [85]. From this structure, novel derivatives might be produced by introducing different functional groups at R_1 and R_2. In this way, two sub-groups can be generated: benzylpiperazines **(Figure 1.20b)** and phenylpiperazines **(Figure 1.20c)** that are mainly characterized by an aromatic ring attached to the piperazine moiety

to which BZP and TFMPP are included, respectively [85]. Some other substances belonging to the sub-groups are presented in **Figure 1.21** where R_1= -H, -CH_3 and R_3= -CH_3, -Cl, -F, -OCH_3, -Br [82].

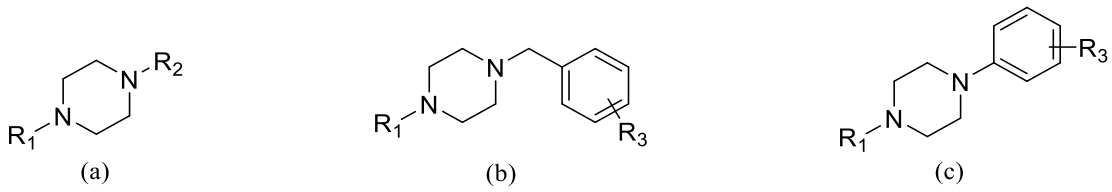
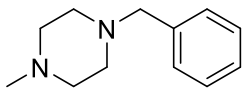
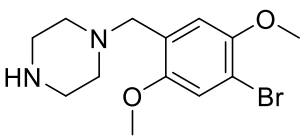


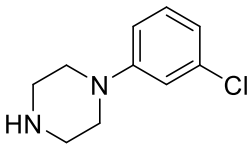
Figure 1.20 | General structures of (a) piperazines, (b) benzylpiperazines and (c) phenylpiperazines, where R_1, R_2 and R_3 are the substituents [85].



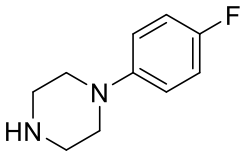
(a) MBZP



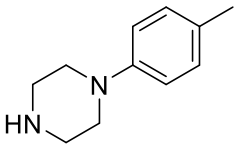
(b) 2C-B BZP



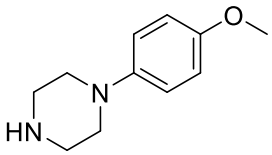
(c) 3-CPP



(d) 4-FPP



(e) 4-MePP



(f) 4-MeOPP

Figure 1.21 | Examples of piperazine derivatives for each sub-group: benzylpiperazines (a and b) and phenylpiperazines (c, d, e and f) [82].

1.3.6 Plant-based substances

This class of compounds comprehends three different plants: khat, kratom and *salvia divinorum.* It is important to mention that any of them are listed in the United Nations Conventions (1961 and 1971). However, some countries have adopted some control measures to restrict their use among their population. For khat, cathinone and cathine, which make part of its chemical composition are already under international control [80].

Khat named *Catha edulis* is a plant commonly found in the Horn of Africa and the Arabian Peninsula [86], which has an impact on the social and cultural life of the communities [87]. In other words, chewing its leaves seem to be a regular practice among the indigenous people, as it induces pleasure and stimulant effects as well as contributes to enhance social interaction [88]. This plant has been used as a drug of abuse and is available under the following street names: "Kat", "Qat", "Chat", among others [89].

Concerning its composition, the shrub contains essentially an array of alkaloids, flavonoids and glycosides [86]. Of these chemical components, cathinone[f] **(see Figure 1.4a)** and cathine[f] **(Figure 1.22)** are the most important and they are responsible for the amphetamine-like effects [87]. Cathinone, the largest active constituent, releases catecholamines immediately after the leaves are chewed; this event can explain the stimulant actions,

[f] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

which are known to be affected by the freshness of the leaves [90]. On the other hand, cathine bears the responsibility for the undesirable effects that might occur [91]. In this way, fresh leaves are preferred owing to their higher content of cathinone to cathine, hence leading to minor side effects [91].

In terms of adverse effects, it is worth noting that khat promotes an increase in blood pressure and heart rate. In case of long-term use, the manifestations might be more severe, such as neurological disorders, psychosis, violence, organ damage, among others [4]. In addition, the effects can remain up to 3 hours [89].

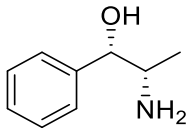


Figure 1.22 | Cathine [87].

Kratom also known as *Mitragyna speciosa* is a tropical tree indigenous to Southeast Asia (*i.e.* from Thailand, Malaysia, Myanmar, The Philippines and New Guinea), which has been disseminated globally through the Internet drug websites [92]. Traditionally, the natives used to chew the leaves of kratom to make them boost their energy, besides that it was consumed as a replacement of opium and to control opioid withdrawal symptoms by chronic users [93,94].

The main active components found in kratom are mitragynine **(Figure 1.23a)** and 7-hydroxymitragynine **(Figure 1.23b)** [95]. These alkaloids compounds by binding the opioid μ-receptor can cause stimulation and sedation effects [95], which may differ in the dosage [96]. Thus, small portions are associated with stimulant results, whereas large quantities (10-25 g of dried leaves) [97] lead to soothing reactions, which can be similar to that of morphine [98]. The users might experience the following effects [93]:

- increased physical energy, alertness and sociable behaviour (low doses);
- nausea, dizziness, sweating, euphoria, increased urination and loss of appetite (high doses) – these symptoms emerge within 5 to 10 minutes after ingestion and may remain up to 5 hours. It is worth noting that the consumption of kratom can lead to addiction. In addition, it is reported several cases of psychosis and some fatalities due to overdose.

On the illegal drug market, kratom is regularly termed "Krypton", which involves a combination of caffeine and O-desmethyltramadol [4,86]. However, other street names can also be identified, such as "Thang", "Thom" and "Ketum" [4]. In terms of administration, ingestion seems to be the major mode of intake [4].

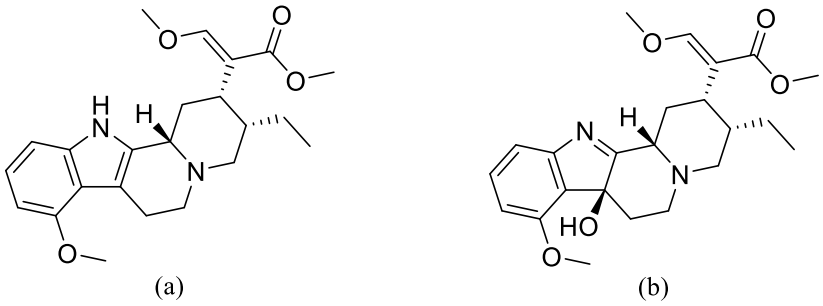


Figure 1.23 | (a) Mitragynine and (b) 7-hydroxymitragynine [95].

Salvia divinorum or salvia is a plant native to the state of Oaxaca (Mexico) [99], which is known to contain hallucinogenic properties [100]. Originally, it was described for medical, spiritual and religious purposes [101]. The main active component found in this plant is termed salvinorin A (a non-alkaloid agent) **(Figure 1.24)** [86] that might contribute to its psychedelic effects, such as dizziness, distortions of objects, hallucinations, decreased ability to interact with the surroundings and overlapping realities [102]. These effects occur within 5 to 10 minutes after ingestion [102] (*i.e.* by chewing, swallowing or smoking) [103]. In other words, the leaves can be chewed, taken as an infusion or smoked [103]. Depending on the mode of use, the reactions might have a short or long-term action (*i.e.* from a few minutes to 2 hours) [101]. On the recreational market, salvia is available under the following street names: "Diviner's Sage", "Sally-D", "Magic Mint", among others [4].

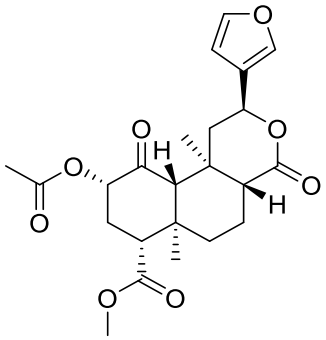


Figure 1.24 | Salvinorin A [86].

1.3.7 Miscellaneous group

This group comprises substances that do not fit into the aforementioned categories. In this sense, they can be divided according to their psychoactive effects: stimulants (*e.g.* **aminoindanes**), dissociatives (*e.g.* **phencyclidine-type substances**), hallucinogens (*e.g.* **tryptamines**), opioids (*e.g.* **fentanyls**) and sedatives/hypnotics (*e.g.* **benzodiazepines**).

Aminoindanes (AIs) possess analgesic and bronchodilating features [4]. The derivatives emerged on the drug market are fabricated based on the structure of 2-AI **(Figure 1.25a)** [104], which is an analogue of amphetamine **(see Figure 1.4b)** [105]. In this way, the inclusion of functional groups [106] to the 2-AI backbone structure may produce a diversity of chemical structures, such as [105]:

- 5-IAI **(Figure 1.25b)** with the addition of an iodine atom at the position 5 on the aromatic ring;
- MMAI **(Figure 1.25c)** and MEAI **(Figure 1.25d)** characterized by methoxy and/or methyl groups;
- MDAI **(Figure 1.25e)** and MDMAI **(Figure 1.25f)** includes a methylenedioxy bridge and/or *N*-alkylation.

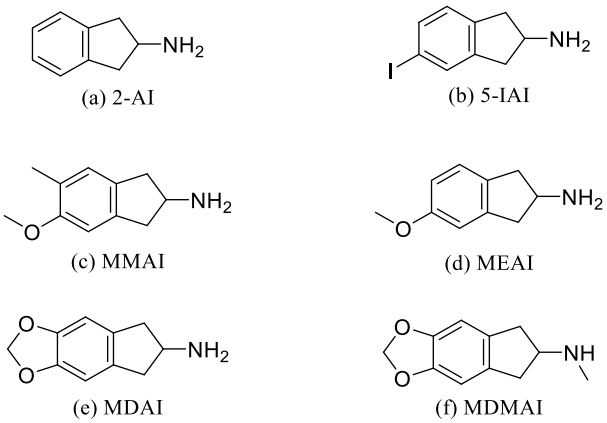


Figure 1.25 | (a) The 2-AI backbone structure, (b), (c), (d) and (f) represent some examples of AIs [104,105].

Phencyclidine-type substances exhibit a comparable structure to ketamines **(see section 1.3.3)**; therefore making also part of the arylcyclohexylamines group [27]. PCP[g] **(see Figure 1.8b)** contains potent analgesic and dissociative properties [107]. At the beginning, this drug was developed as an intravenous anaesthetic; however, their users experienced adverse effects (*e.g.* agitation, psychosis and delirium), which lead to the withdrawal from clinical use [62]. Later, PCP emerged on the recreational market as a white powder, tablet or liquid forms under the street names: "Angel Dust", "Ozone", "Rocket Fuel", among others [108]. Some PCP analogues are illustrated in **Figure 1.26** [62] . The effects produced may range between stimulant and depressant depending on the administration route, as well as dosage [109]. According to the way of administration, it can be snorting, swallowing and smoking, being the latter one the most frequent method of use [108]. In terms of dosage, low portions (*i.e.* 1 to 5 mg) cause apathy, blank stare, detachment feelings, and loss of coordination [108]. On the other hand, high quantities may result in hallucinations, increased blood pressure and heart rate [108].

Figure 1.26 | Examples of PCP analogues: (a) 3-MeO-PCP and (b) 3-HO-PCP [62].

Tryptamines (Figure 1.27a) [110] came from the decarboxylation of the amino acid tryptophan **(Figure 1.27b)**; both share an indole ring and aminoethyl group at position 3 [74,111]. Chemical modifications in the skeleton structure of tryptamine may contribute to novel derivatives, which might affect the physical and mental health of their users [112]. Tryptamines became famous thanks to the publication of the book "TIHKAL: The continuation", who the author is Alexander Shulgin [111]. These compounds are sold in the form of powders or tablets, which can be consumed by ingestion, snorting, inhalation and smoking [74].

[g] Controlled under the Schedules of the Convention on Psychotropic Substances of 1971 [80]

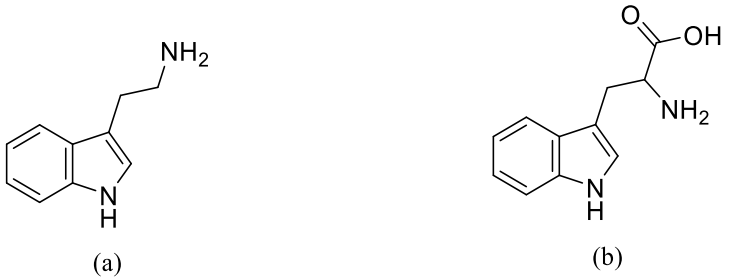


Figure 1.27 | Structures of (a) tryptamine and (b) amino acid tryptophan.

In terms of classification, they can be split into three different groups, which are illustrated in **Figure 1.28** [112]:

- tryptamines without any modifications on the indole ring (*e.g.* DET[g] and DMT[g]);
- tryptamines with an alteration at the position 4 on the indole ring (*e.g.* 4-OH-DiPT and 4-MeOH-MiPT);
- tryptamines comprising a substituent at the position 5 on the indole ring (*e.g.* 5-MeO-DET and 5-MeO-DMT).

The recreational use of these substances is associated with psychotropic events and a high exposure to overdose [112].

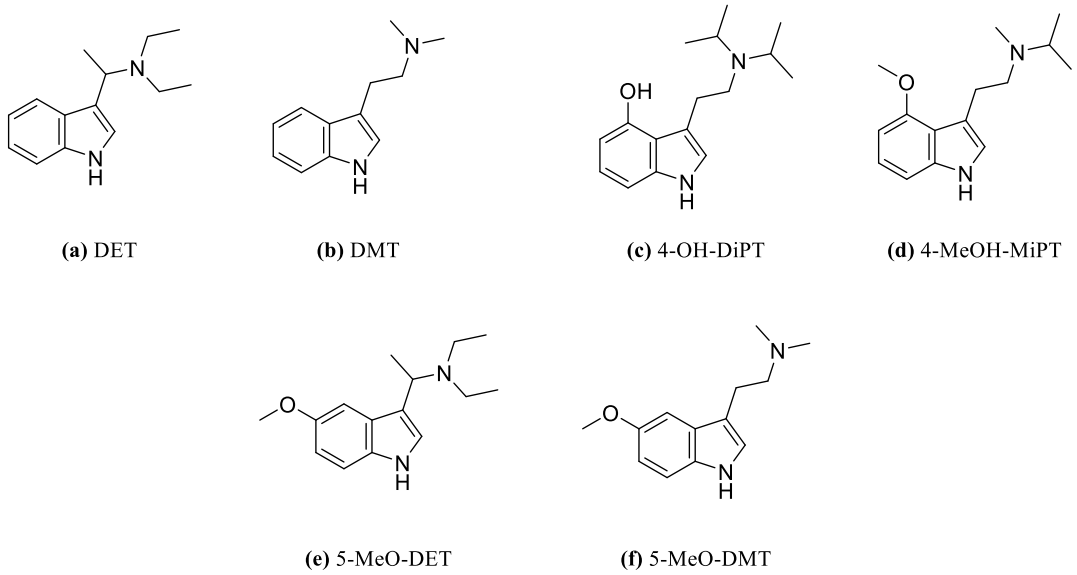


Figure 1.28 | Tryptamines classification: unsubstituted indole ring (a and b), 4-substituted (c and d) and 5-substituted (e and f) [112].

In 1960s, **fentanyls (Figure 1.29a)** [113] were manufactured with the purpose to alleviate acute and chronic pain, which was possible due to their ability to bind μ-opioid receptors producing anaesthetic effects [114]. It is worth noting that fentanyls are more powerful (*i.e.* 100 times) in comparison with morphine [114], thus they became a valuable analgesic substitute in clinical use [114], thanks to their rapid onset and short duration of action [115]. In this way, the outset of their recreational abuse might be connected with the easy access to these substances by health professionals [114]. Thereafter, fentanyls have increased in popularity and consequently, expanded to the general public [114]. The illicit use may lead to overdose deaths, as they possess a low lethal dose, which means that an intake of a few milligrams is fatal for their users [114] – respiratory arrest and pulmonary

edema might emerge [116]. Despite the aforementioned consequences, other side effects not as severe may occur, such as sedation, dependence, breathing disturbances, nausea, dizziness, fatigue, among others [116]. Concerning administration routes, they can be intravenously, intramuscularly or transdermal administered [117]. Chemical modifications by introduction of functional groups on the piperidine and/or phenyl ring for example, might produce novel analogues **(Figure 1.29)** [113,117]. Currently, the previous derivatives are under international control [80].

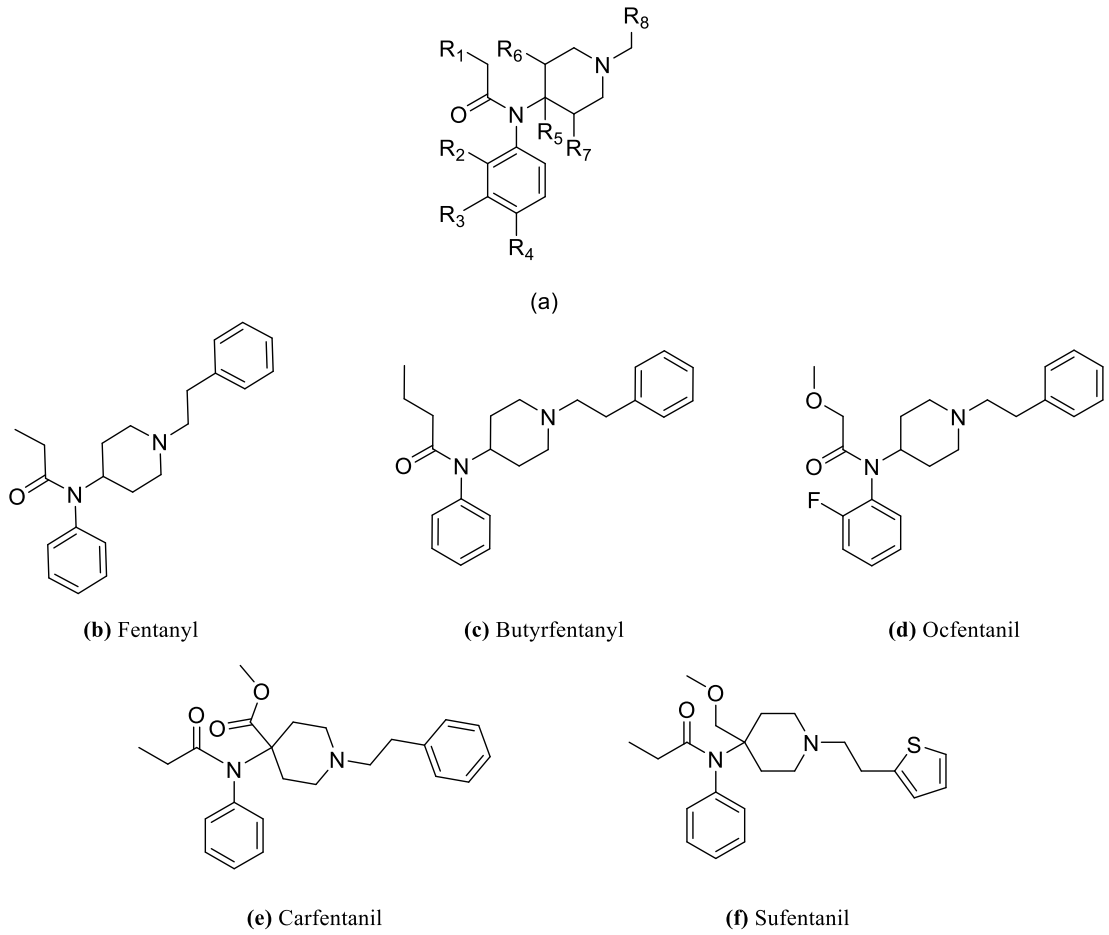


Figure 1.29 | (a) General structure of fentanyls, where R_1,R_2,R_3,R_4, R_5, R_6, R_7 and R_8 represent the substituents, (b), (c), (d) and (e) are fentanyl analogues [113,117].

Benzodiazepines are referred to as central nervous system (CNS) depressants, which replaced barbiturates frequently recommended to treat anxiety and sleep disorders, owing to their minor adverse effects and low risk of provoking an overdose [118]. Nevertheless, this class of drugs exhibits a potential of misuse and dependence [119]. Their abuse comprises the combination with other controlled substances, such as opiates, methadone and cocaine [118]. It is worth noting that opiates along with benzodiazepines could promote increased euphoric effects [118]. In addition, these drugs mixed with alcohol might lead to several cases of poisoning. In this way, fatal intoxications are more likely to occur when benzodiazepines are abused not as single substances [120]. The emergence of these substances as designer drugs started in 2011 with the appearance of pyrazolam **(Figure 1.30)** in forensic cases [119,121]. Later on, novel derivatives came out on the drug market; they are mainly characterized by slightly modifications in their chemical structures [120].

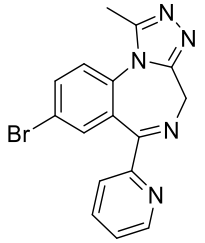


Figure 1.30 | Pyrazolam [119].

The backbone structure of benzodiazepines consists of a diazepine ring fused to a benzene ring [122]. In general, to this diazepine ring a phenyl ring is attached [122]. Hence, based on tiny alterations, it is possible to distinguish them into several sub-classes **(Figure 1.31)**. The manufactured analogues were founded on 1,4-benzodiazepines [120]. Therefore, the addition of a cyclic system (*e.g.* a triazole, imidazole and oxazole ring) to the previous chemical structure could give rise to triazolobenzodiazepines, imidazobenzodiazepines and oxazolobenzodiazepines, respectively [122]. Furthermore, the replacement of the benzene ring with a thiophene ring (*i.e.* thienodiazepines) and lastly, the trienotriazolodiazepines that possess a thiophene and a triazole ring fused to the diazepine ring [122]. These synthetic benzodiazepines are available in an array of dosage forms, such as powders, capsules and tablets [120]. In terms of effects, they may range between anxiolytic, sedative and hypnotic side effects, which differ in the dose [119].

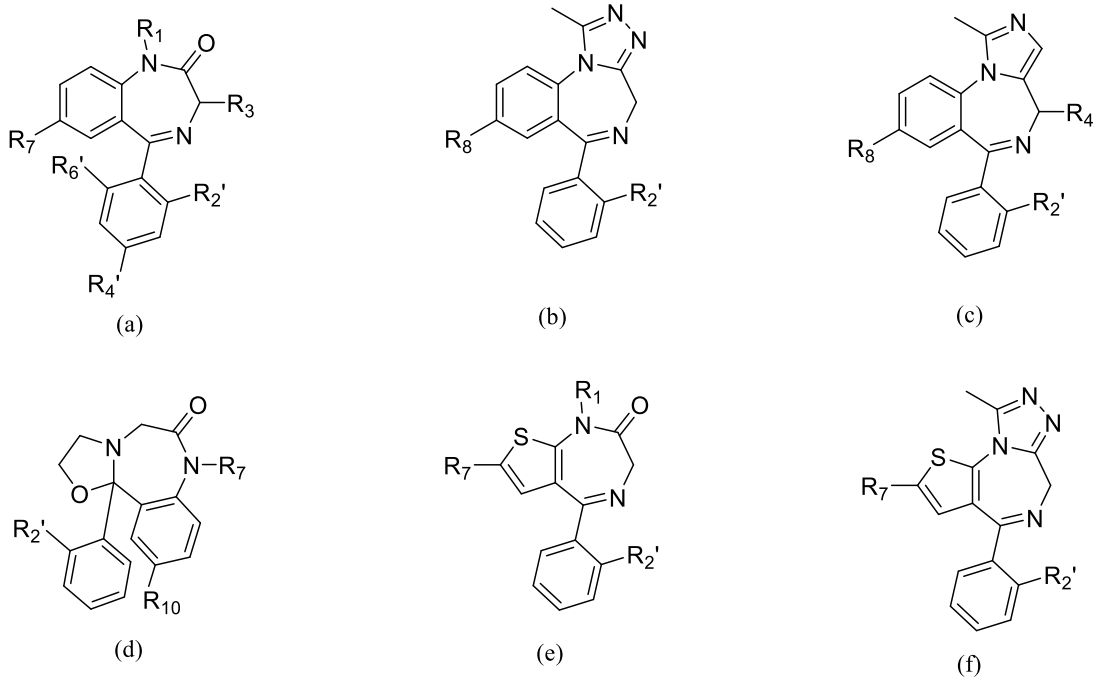


Figure 1.31 | Some benzodiazepines sub-classes: (a) 1,4-benzodiazepines, (b) triazolobenzodiazepines, (c) imidazobenzodiazepines, (d) oxazolobenzodiazepines, (e) thienodiazepines and (f) trienotriazolodiazepines. R represents the substituents [122].

1.4 Identification Methods of NPS

In forensic laboratories, gas chromatography-mass spectrometry (GC-MS) has been referred to as a gold standard technique for the identification of traditional drugs [123]. However, the appearance of NPS on the drug market revealed the method to be incomplete in pinpointing these compounds [123]. In other words, the use of GC-MS databases is often restricted due to the lack of mass spectra concerning these novel substances [123].

Besides that, the structural similarity of these psychoactive compounds is not always evident, which might lead to unreliable interpretation when the analysis is only based on an electron impact-mass spectrometry (EI-MS) spectrum [123]. Moreover, the use of EI as an ionisation technique makes their determination challenging, especially when the produced ions correspond to low intensity fragments [123]. Therefore, it is necessary to resort to more sophisticated techniques to address the previous issues. In this sense, high-resolution mass spectrometry (HRMS) can be an alternative in the analysis of narcotics, since it provides an accurate mass of the analytes, allowing to elucidate their molecular formulae and as consequence, the list of possible candidates can be reduced to one or to a limited number of compounds, which can be further investigated in available databases [124]. In recent years, LC tandem MS or LC high-resolution (HR)-MS such as, the hybrid quadrupole time-of-flight (QTOF), became commonly used for the identification, determination and quantification of NPS [125].

After the sample preparation, the extract is separated in a liquid chromatogram. Of note, LC is characterised by its versatility owing to its ability to separate volatile and non-volatile compounds [126]. Improvements on this technique led to columns containing particle sizes lower than 2 μm [125] and a higher temperature tolerance, which contributed to an enhanced separation of the substances and a shorter analysis time, these novel features gave rise to the ultra-high performance LC, the so-called UHPLC [127]. Before the analytes enter on the mass analyser, they are ionised in the LC-MS interface (*i.e.* ion source), producing gas-phase ions. On the framework of this thesis, electrospray ionisation (ESI) was employed, since it is suitable for both small and large molecules within a broad polarity range [125]. This ionisation technique is produced by applying a strong electric field under atmospheric pressure to the liquid, thus generating charged droplets. Depending on the ionisable sites, it is possible to observe substances carrying multiple charges, which is more likely to occur in proteins [123]. On the other hand, some molecules do not comprise any ionisable sites, therefore the formation of adducts (*e.g.* sodium, potassium and ammonium) is prone to take place [123]. It is important to notice that ESI is described as a soft ionisation method, in this way nearly no ion fragmentation is present during the ionisation process [125]. Hence, it is expected to obtain protonated molecular ions $(M+H)^+$, sodium and potassium adducts or deprotonated molecular ions $(M-H)^-$, formate and acetate adducts, which rely on the operation mode positive and negative, respectively [125]. It is worth noting that most of NPS are positive-ionised [123]. In light of above, ESI may lead to insufficient information on fragmentation [123]. To surpass this drawback, the fragmentation might be forced by a fragmentor voltage, causing the ions to collide with other molecular species (*e.g.* gas molecules), consequently the energy generated will promote the fragmentation to ensue [123]. This technique is termed in-source fragmentation, also known as in-source collision-induced dissociation (in-source CID) [123]. Based on the fragmentor voltage, the degree of in-source fragmentation can be determined [123].

In a QTOF, the quadrupole (*i.e.* a mass filter) is composed by four parallel rods where a voltage is applied producing an electric field that allows the selection of ions with a particular mass-to-charge ratio (*m/z*) and consequently, their routing to the detector [123]. Before that, the ions undergo fragmentation that occurs in the collision cell by the contact with an inert gas (*e.g.* nitrogen, argon) [123]. According to the collision energy (CE), the dissociation of the analyte may drive to different ion intensities which are hinge on the strength of each bond in a molecule [123]. Therefore, the fragmentation may result in the source or in the collision cell [123]. It is important to highlight that insights into fragmentation patterns of specific NPS drug classes are crucial for a proper structure elucidation of the compound at hand [123]. As mentioned previously, the fragmented ions move along a flight tube with a reflectron that compensates the dispersion of kinetic energy of those ions with the same *m/z*, so that the ions can reach the detector at the same time, generating the mass spectrum [128]. Of note, the smaller ions arrive at the detector earlier [128].

In terms of data acquisition, HRMS can operate in data-independent acquisition (DIA) **(Figure 1.32a)** and data-dependent acquisition (DDA) **(Figure 1.32b)** modes [128,129]. Thus, in DIA all ions recorded in the mass

analyser are further fragmented in the collision cell, whereas DDA mode works differently, which means that for this technique a specific precursor ion is selected instead [129]. The former approach is more applicable to high-resolution instruments and provides with retrospective data interrogation allowing the analysts to reanalyse a sample without the need to reinject in the system [129]. Besides that, it has the ability to acquire data by switching between low- and high-energy in the same run [129]. DIA allows to obtain accurate mass of the precursor and product ions for structure characterisation. This acquisition mode is also referred to as all-in-one, the so-called MS^E, where E stands for collision energy and is developed by Waters Corporation, Milford, Massachusetts, United States of America [128,129]. The use of DIA might compromise the spectra interpretation, since it does not select a particular precursor ion, thus the association of the product ions with a correct precursor ion may be complex [129]. However, this pitfall is improved when UHPLC is combined with QTOF contributing to a better separation [128]. In conclusion, HRMS contributes with putative structural elucidation that might be confirmed using nuclear magnetic resonance (NMR) spectroscopy, especially when positional isomers are involved [129]. In addition, LC-ESI-QTOFMS can be applied as an add-on method to GC-EI-MS, which is routinely operated in forensic laboratories [123]. In this sense, the mechanisms of fragmentation under different ionisation techniques will promote different spectra, resulting in supplemental information on fragmentation, which is essential for the determination of NPS [123].

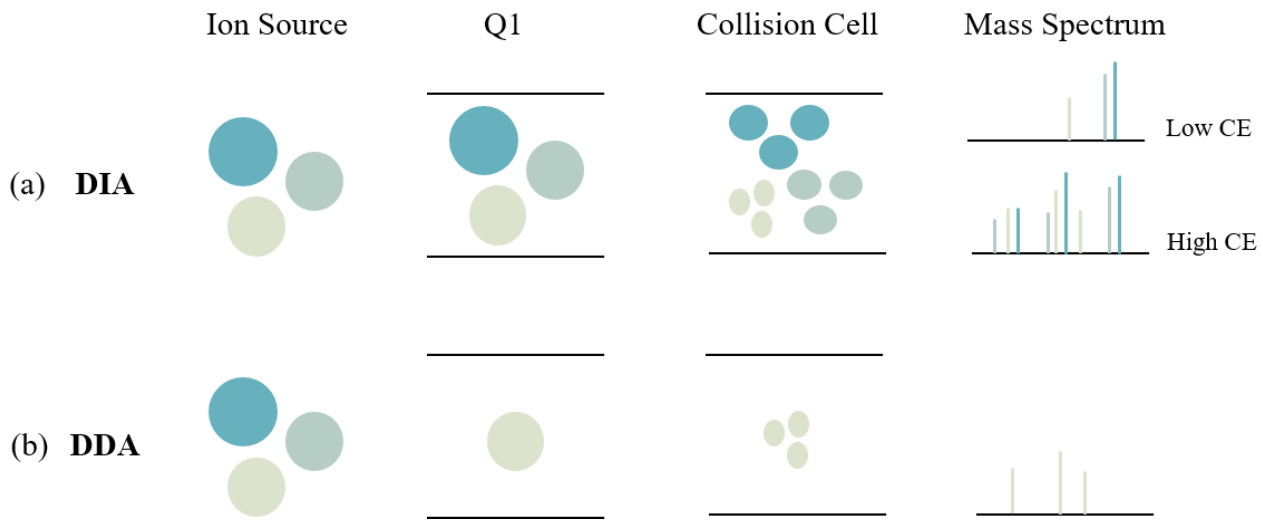


Figure 1.32 | DIA and DDA acquisition modes.

1.5 HighResNPS database

In 2016, an Internet mass spectral database for liquid chromatography-mass spectrometry (LC-MS) was created by a group of Danish researchers from the Department of Forensic Medicine of the University of Copenhagen and Aarhus University. The purpose of this database is trying to keep the libraries updated for screening of NPS, which is mostly challenging due to the continuous growth of these drugs into the black market and lack of reference standards. HighResNPS **(Figure 1.33)** is a crowd-sourced and free-of-charge database that has the contribution of a diversity of laboratories spread around the world, however is only designed for a closed user group within the forensic field [130,131]. The different contributors define their experimental setup and location, so that new entries can be added [132]. The records should contain the exact mass of protonated molecular ions with exact mass of up to three highest intensity fragment ions, retention time (R_t), International Chemical Identifier (InChI) key, systematic name (IUPAC) and respective drug class [132]. In addition, the acquired mass spectra can also be included [132]. Of note, the database can further be exported as an Excel spreadsheet, which makes it useful, distinguishable from other NPS libraries and compatible for HR-MS screening platforms such as, Agilent QTOF MS, Bruker QTOF MS, Waters QTOF MS or Thermo Orbitrap MS [130].

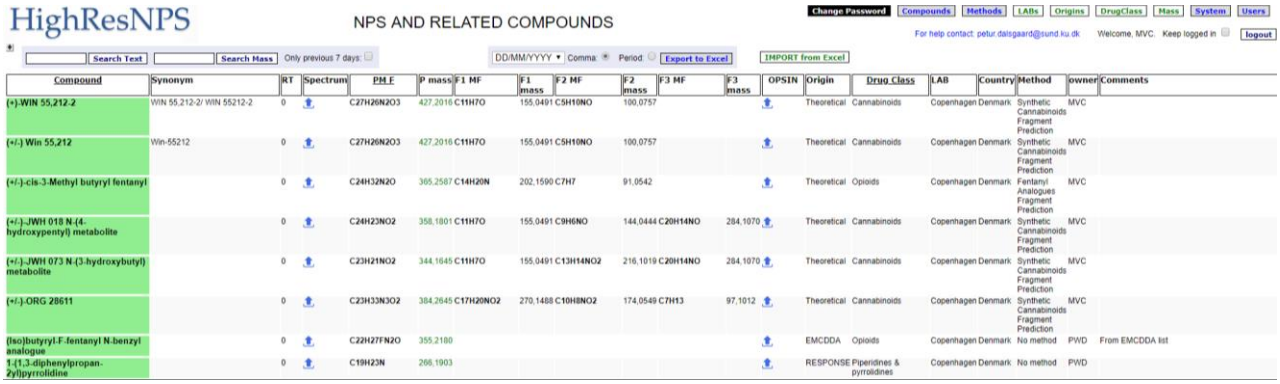
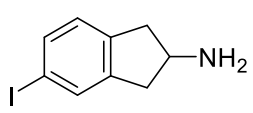


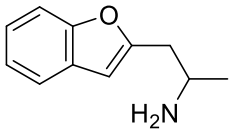
Figure 1.33 | HighResNPS overview: front-page.

Currently, there are 19 countries (Australia, Belgium, Canada, Croatia, Denmark, Finland, Germany, Greece, Hong Kong, Italy, New Zealand, Norway, Portugal, Scotland, Spain, Switzerland, The Netherlands, The United Kingdom and The United States) involved in the database project. However, only a few of them are active users and have contributed with relevant data in the last months of 2018 and at beginning of 2019, especially Australia, Denmark, Finland, Norway and the United Kingdom.

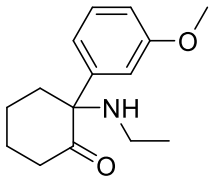
Regarding the classification drug system employed on the database, there are some slight variances in comparison with the UNODC categorization. The compounds can be grouped into the following categories: aminoindanes, arylalkylamines, arylcyclohexylamines, benzodiazepines, cannabinoids, cathinones, indolalkylamines, opioids, phenethylamines, piperazine derivates, piperidines & pyrrolidines, plants & extracts and unknown **(Figure 1.34)**. The latter class corresponds to substances that do not fall into any of the previous groups.



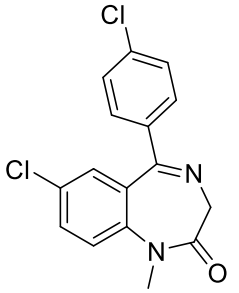
(a) 5-IAI



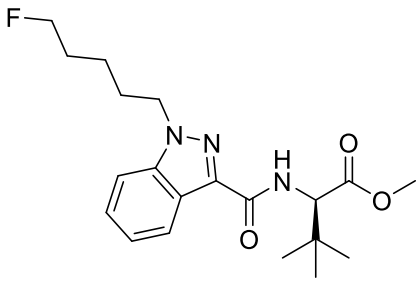
(b) 2-APB



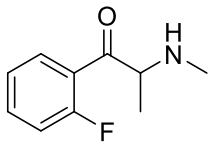
(c) Methoxetamine



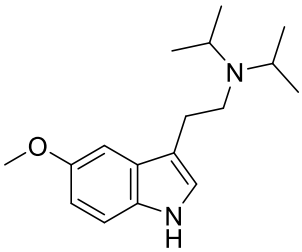
(d) 4-Chlorodiazepam



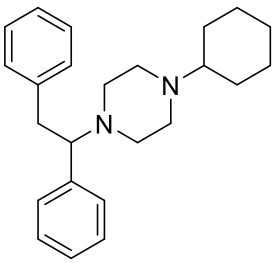
(e) 5F-ADB



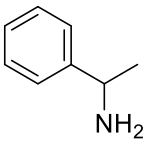
(f) 2-FMC



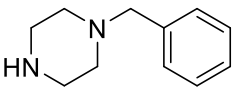
(g) 5-MeO-DiPT



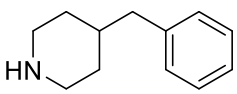
(h) MT-45



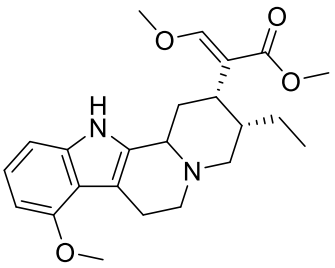
(i) 1-PEA



(j) BZP



(k) 4-Benzylpiperidine



(l) Mitragynine

Figure 1.34 | Drug classes on HighResNPS database with a characteristic compound: (a) aminoindanes, (b) arylalkylamines, (c) arylcyclohexylamines, (d) benzodiazepines, (e) cannabinoids, (f) cathinones, (g) indolalkylamines, (h) opioids, (i) phenethylamines, (j) piperazine derivates, (k) piperidines & pyrrolidines and (l) plants & extracts.

2 AIMS OF THE STUDY

The main purpose of this thesis is to improve and test a universal online library (HighResNPS) for NPS when reference standards are not available using UHPLC-QTOF-MS (ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry). In order to achieve this, the project was divided into two parts, which are briefly described below.

Part I – HighResNPS

- To monitor six drug websites: chem.eu, drugs-forum.com, erowid.org, EWS forum, forendex and NMS labs, so that novel compounds could be added on the database. The obtained data consists of non-experimental parameters (*e.g.* exact parent mass, molecular formula, InChIKey, IUPAC name and drug class);

- To validate the database by including new features – this comprehends the correction of parent and fragment ion masses, as well as finding the missing fragment formulae and lastly, deleting all the double entries reported by RESPONSE and EMCCDDA libraries (*i.e.* the ones containing only parent masses);

- To create two sub-libraries for synthetic cannabinoids and fentanyl analogues comprising information concerning the predicted fragment ions.

Part II – Screening

- To develop and evaluate the applicability of a targeted screening method for 14 NPS in seized material using UHPLC-QTOF-MS and HighResNPS as an online mass spectral database.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

All solvents were liquid chromatography-mass spectrometry (LC-MS) grade. Methanol, water, formic acid and acetonitrile were purchased from Fisher Scientific (Leicestershire, United Kingdom), whereas ammonium formate was supplied by Sigma-Aldrich (Steinheim, Germany). The TOF solution consisted of a water:methanol:formic acid (74:25:1, *v/v/v*) mixture and an internal standard (carbamazepine at 1 mg/mL) prepared by the Section of Forensic Chemistry (Copenhagen, Denmark).

3.1.2 Seized material

36 seizures were confiscated by the Danish authorities during the years 2015 and 2019. The analysed material consisted of powders, tablets and herbal matrices.

3.2 Screening method

3.2.1 Sample preparation

50 mg of seized material was extracted in 5 mL methanol on a rotator PTR-35 vertical multi-function (Grant Instruments, Cambridge, United Kingdom) for 10 min followed by centrifugation on a ROTOFIX 32 A (Hettich,Tuttlingen, Germany) for 5 min at 3000 rpm. The prepared solution was further filtrated and diluted in two steps to a factor of 500 using the TOF solution.

3.2.2 Ultra-performance liquid chromatography

The chromatography was performed using a Waters ACQUITY UPLC system from Waters Corporation (Milford, Massachusetts, United States of America). The autosampler injected 3 µL of extract into an ACQUITY UPLC HSS C18 column (2.1 mm x 150 mm, 1.8 µm) maintained at 50 ºC in a column oven.

LC separation of the samples was performed through a gradient profile of mobile phase A and B solutions, consisting of 5 mM aqueous ammonium formate buffer adjusted to pH 3 with formic acid and acetonitrile with 0.1% formic acid *(v/v)*, respectively. The gradient was 0 min to 0.5 min: 13% (B); from 0.5 min to 10 min: 13% to 50% (B); from 10 min to 10.75 min: 50% to 95% (B); from 10.75 min to 12.25 min: 95% (B) and from 12.25 min to 15 min: 95% to 13% (B). The flow rate was 0.4 mL/min and the total run time was 15 min.

3.2.3 Mass spectrometry

An orthogonal accelerator quadrupole time-of-flight mass spectrometer, Xevo G2-S QTOF (Waters MS Technologies, Manchester, United Kingdom) was used for screening of NPS in positive electrospray ionization (Z-spray) mode. The following settings were employed: nebulization gas 800 L/h, with a temperature of 400 ºC; cone gas flow 20 L/h; source temperature 150 ºC; capillary voltage 800 V; cone voltage 25 V and argon was used as collision gas. Data was recorded in DIA with elevated collision energy (MS^E). The low collision energy (CE) was set at 4 eV and high CE ramped from 10 to 40 eV. The acquisition time was the entire run, with a scan time of 0.200 s. The mass range was from *m/z* 50 to *m/z* 950. For resolution measurement and

guarantee of reproducibility and accuracy, a LockSpray™ was applied. Lock mass was used with leucine enkephalin as a reference mass at *m/z* 556.2766.

3.3 Software

3.3.1 Data Analysis

The data acquisition and data processing were performed with the UNIFI Scientific Information System (Waters) version 1.9.2 (Waters Corporation, Milford, Massachusetts, United States of America). The UNIFI software ensures that only relevant ions are selected and associated with a specific component. In addition, the elemental composition of the substance under investigation is automatically calculated and searched against internal libraries and databases using both precursor and fragment ion data (*e.g.* HighResNPS database). It also provides *in-silico* fragmentation information, assigning plausible or likely structures to the observed ions, which is helpful when standards are not available or difficult to purchase. To identify the substances the *m/z* tolerance for both precursor and fragment ions was 3 mDa.

3.3.2 Chemical Drawing Software

All chemical structure generation and fragmentation prediction were carried out by *ChemBioDraw Ultra 16.0* (PerkinElmer, Waltham, Massachusetts, United States of America).

3.3.3 OPSIN

This platform was used to generate the chemical name into a structure. It also comprised the InChIKey associated to that specific molecule, which allowed to make sure that the compound corresponded to the right chemical identifier or the other way around. This open source was useful during the first part of the thesis [133, 134].

3.4 Database

3.4.1 mzCloud™

An Internet database of high-resolution tandem mass spectra containing a freely searchable collection of accurate mass spectra [135]. This platform was used for assisting on the analytical identification and interpretation of the absent fragment ions, fragmentation prediction (part I: HighResNPS database) and in the last part of the thesis for spectra comparison (part II: screening method).

3.5 Additional libraries

3.5.1 TiHKAL

An online library was used to search for the absent IUPAC names, InChIKey identifiers and possible synonymous for a specific substance (HighResNPS database validation process) [136].

3.5.2 RESPONSE and EMCDDA

RESPONSE [137] and EMCDDA libraries provided initially new entries to HighResNPS database. Both contributed with chemical information such as, molecular formula of the substance of interest, InChIKey, IUPAC name and drug class.

4 RESULTS AND DISCUSSION

In this section, the work conducted will be described in detail. In order to provide an overview of the results obtained, the study was divided into two main parts, the first one is related to optimizing the database and the second to testing it to screen for NPS.

4.1 Part I – HighResNPS database

The exponential growth of NPS on the illegal drug market forced the forensic laboratories to find a different approach to screen these designer drugs. NPS represent a challenge for analysts that are willing to give a reliable answer to the cases at hand; this is due to the limited information on the pharmacological and toxicological effects. Besides that, the slight modifications in their chemical structures may compromise the analysis, in this way leading to a non-accurate interpretation owing to the structure similarity and the lack of reference standards, which are not always readily available. In order to try to tackle these issues, a group of Danish researchers had the idea of creating a database named HighResNPS.com. This database presents unique features that may distinguish it from commercial databases. It comprehends new psychoactive substances as well as related compounds reported from an array of laboratories around the world. In this way, the same compound can have more than one entry, which contributes for self-validation, in other words it is possible to make sure that the data provided is correct. As mentioned previously, 19 countries are involved in the project: Australia, Belgium, Canada, Croatia, Denmark, Finland, Germany, Greece, Hong Kong, Italy, New Zealand, Norway, Portugal, Scotland, Spain, Switzerland, The Netherlands, The United Kingdom and The United States. However, only a few of them are in fact, active users. Since the last months (October, November and December) of 2018 to the beginning of 2019, merely Australia, Denmark, Finland, Norway and United Kingdom have been devoted to the database. Currently, HighResNPS comprises 4 analytical instruments: **Agilent QTOF MS** from Adelaide (AU), Oslo (NO) and Trondheim (NO); **Bruker QTOF MS** from Athens (GR), Helsinki (FI) and Aarhus (DK); **Waters QTOF MS** from Copenhagen (DK), IUPA, UJI (ES), Labor Krone (DE) and RCMP-NFLS (CA); **Thermo Orbitrap MS** from Copenhagen (DK), LGC (UK) and Mario Negri laboratory (IT). On the framework of this thesis, two additional sub-libraries: **Fentanyl Analogues** and **Synthetic Cannabinoids Fragment Prediction** from Copenhagen (DK) were included on the database. Both comprehend theoretical information on fragmentation, thus they do not contain any method associated.

The first part of the thesis consisted of improving the database, so that it could be further downloaded and converted to a suspect library for Agilent QTOF MS, Bruker QTOF MS, Waters QTOF MS or Thermo Orbitrap MS. In order to achieve the aim, the former step involved the NPS classification of substances whose drug classes were absent. According to the UNODC categorization, it is possible to distinguish NPS into seven main classes: synthetic cannabinoids, synthetic cathinones, ketamines, phenethylamines, piperazines, plant-based substances and miscellaneous (aminoindanes, phencyclidine-type substances, tryptamines and benzodiazepines) **(see section 1.3)**. Nevertheless, HighResNPS does not follow the exact same criteria. The classification drug system employed includes the following 13 categories: aminoindanes, arylalkylamines, arylcyclohexylamines, benzodiazepines, cannabinoids, cathinones, indolalkylamines, opioids, phenethylamines, piperazines derivatives, piperidines & pyrrolidines, plants & extracts and unknown. Arylalkylamines are composed by phenylalkylamines (*e.g.* phenethylamines) and indolalkylamines (*e.g.* tryptamines); arylcyclohexylamines consist of ketamines and phencyclidine-type substances; opioids comprise compounds of natural, semisynthetic, synthetic and endogenous origin that interact with opioid receptors, of which fentanyl analogues belong; piperidines & pyrrolidines are not considered in the UNODC NPS categories; unknown does not mean the compounds are unrecognized, instead they do not fall into any of the prior classes and the remaining groups do not suffer any modifications. Regarding arylalkylamines, this drug class comprehends a broad number of

compounds, especially phenethylamines and indolalkylamines that contain their own specific group on the database. In order to simplify, all substances that do not present an identical chemical structure to phenethylamines and indolalkylamines are considered part of arylalkylamines **(see section 1.5; figure 1.29b)**.

At the beginning of this work, the database contained 1671 entries **(Figure 4.1a)** of which 706 were missing their NPS classification. Due to the number of multiple entries for the same substance, it is important to refer to these numbers in terms of compounds that were on the database at that time. After downloading the database into an Excel file, it was possible to conclude the presence of 1095 compounds: 7 aminoindanes, 25 arylalkylamines, 19 arylcyclohexylamines, 37 benzodiazepines, 350 cannabinoids, 157 cathinones, 63 indolalkylamines, 104 opioids, 163 phenethylamines, 29 piperazine derivatives, 27 piperidines & pyrrolidines, 19 plants & extracts and 95 unknown **(Figure 4.1b)**.

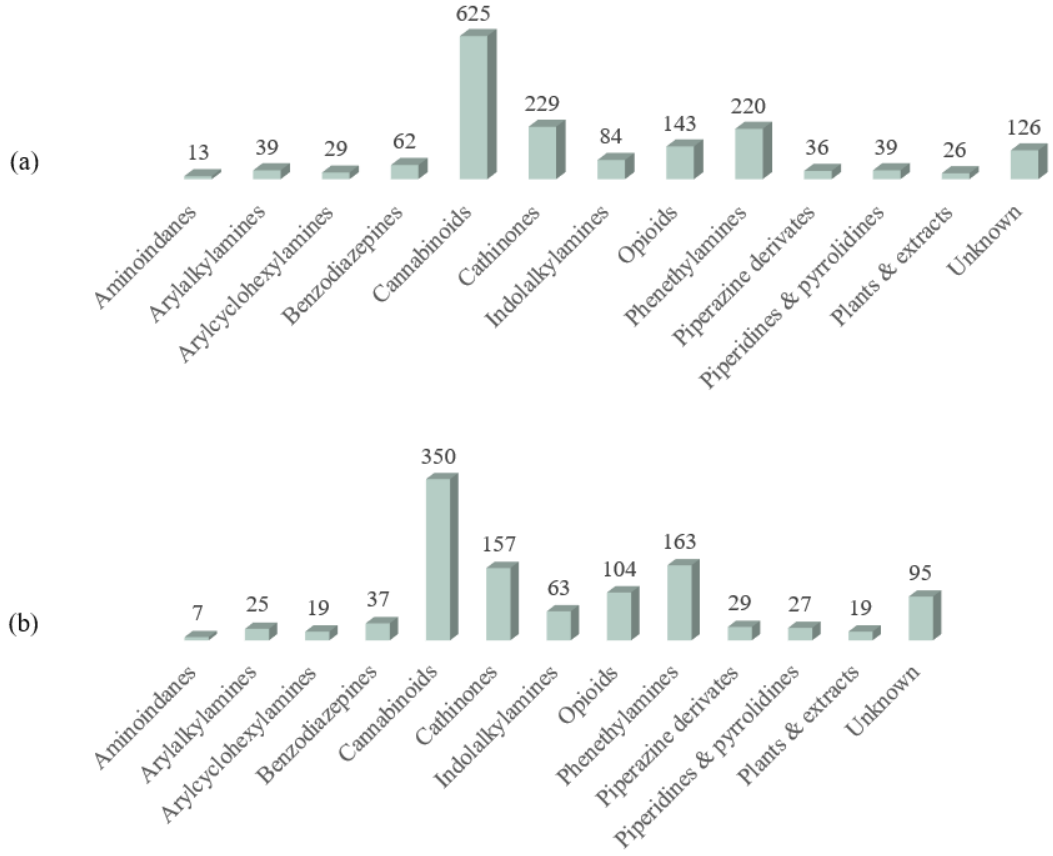


Figure 4.1 | HighResNPS database in October 2018: (a) number of entries and (b) number of compounds per drug class.

A second step was monitoring drug websites, in order to add some emerging compounds or others of interest on the database. This process took place from end of October to middle of November 2018. During this period, 58 novel substances were included on the database **(see Appendix A)** with their respective molecular formula, exact parent mass, drug class, IUPAC name and InChIKey (non-experimental data). Six drug websites were controlled: chem.eu, drugs-forum.com, erowid.org, EWS forum, forendex and NMS labs. Chem.eu [138] is an online market that sells research chemicals all over the world; drugs-forum.com is an informational website regarding drugs and their psychoactive effects [139]; erowid.org is a forum where people describe their experiences with psychoactive plants and chemicals, documenting how and why they are using a specific drug [140]; EWS forum provides information concerning new drug trends among EU member states; forendex is an American website that lists the novel compounds on the drug market [141] and NMS labs offers a comprehensive test

portfolio for NPS to support medical examiners, law enforcement agencies, crime laboratories and healthcare providers [142].

Afterwards, it was necessary to validate the database. The validation process is of paramount importance, since it is essential to ensure that the available data is accurate and there are not flaws that may weaken its potential. In this sense, we realised that mass errors associated with both parent and fragment ions were the most evident along the library, a simple mistake in entering one digit may ruin the entire information. Thus, it was incorporated an integrated calculator, so that the errors would come up automatically in red with the respective difference, as illustrated in **Figure 4.2**. This tool allowed to find 290 entries comprising incorrect values for both parent and fragment ions, which were further revised.

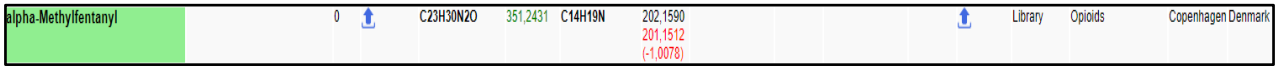


Figure 4.2 | Alpha-Methylfentanyl (opioid): first fragment mass with the right value highlighted in red (201.1512 Da).

Besides that, fragment ion masses as well as their respective molecular formulae were in some cases not present or not matching accordingly. In this way, to find the molecular formulae of the missing fragments, the database was downloaded as an excel file and a pivot table was used. By combining the molecular formula, mass of each fragment and respective ID, it is possible to know how many fragments do not have associated a molecular formula. The following methodology was applied:

- Filter: F1MF, F2MF or F3MF
- Row: F1mass, F2mass or F3mass
- Values: Count of ID

where F1MF, F2MF and F3MF represent the molecular formula of the three most intense fragments; F1mass, F2mass and F3mass are related to the mass of each fragment and the ID corresponds to the number of entries on the database. For each fragment, a table containing the masses was created **(see Appendix B)**, in green are illustrated the ones that were rectified and in red the ones not able to find. Thanks to a new feature integrated on the database, it was possible to type the fragment mass of a certain compound (*e.g.* nitracaine (**1**) was missing the molecular formulae of the first and second fragments – 150.0185 Da and 142.1589 Da, respectively – **Table 4.1**) and click on the entry, its information is displayed. Then, we can look for the mass from a drop-down list, which comprises already the molecular formula associated to that specific fragment mass **(Figure 4.3)**.

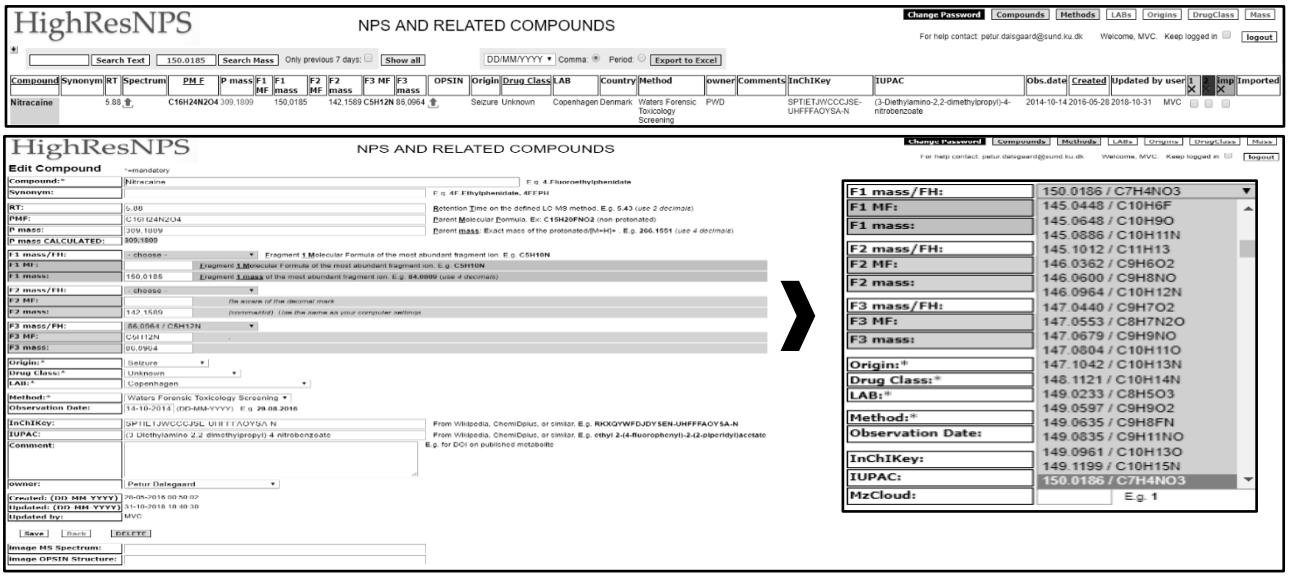


Figure 4.3 | Nitracaine (unknown): list of fragment masses and respective molecular formulae.

For modafinil (**2**), the molecular formula associated to F1mass was not available on the database, thus it was necessary to add it - mzCloud^{TM} was used as an analytical support **(Figure 4.4a)**. The F1mass, F2mass and F3mass of MMMP (**3**) were found from a drop-down list on the database (similar to the one depicted in **Figure 4.3**). The differences between dark red and blue values are slightly small. Likewise in modafinil, the F1mass of alfentanil (**4**) was absent; mzCloud^{TM} provided the molecular formula that we were looking for **(Figure 4.4b)**.

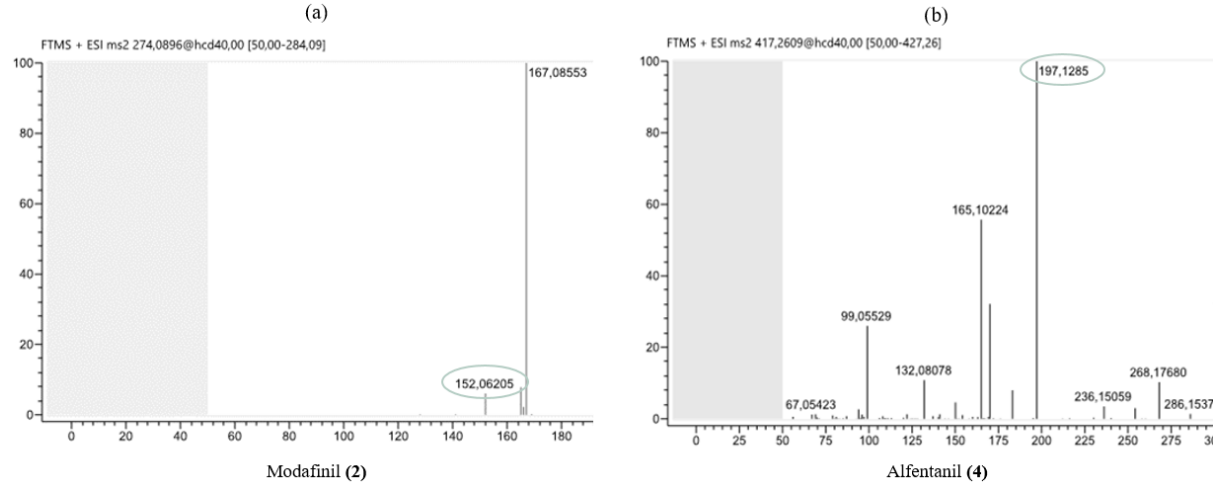


Figure 4.4 | (a) Modafinil and (b) alfentanil: fragment ions (green circle) and their respective molecular formulae were obtained from mzCloud^{TM} spectra.

From (**6**) to (**12**), the molecular formulae were investigated with the support of mzCloud^{TM} **(Figure 4.5)** and when not possible, we resorted to the fragmentation tools of *ChemBioDraw Ultra 16.0*. The latter one was applied to the compounds (**5**), (**7**), (**8**) and (**11**).

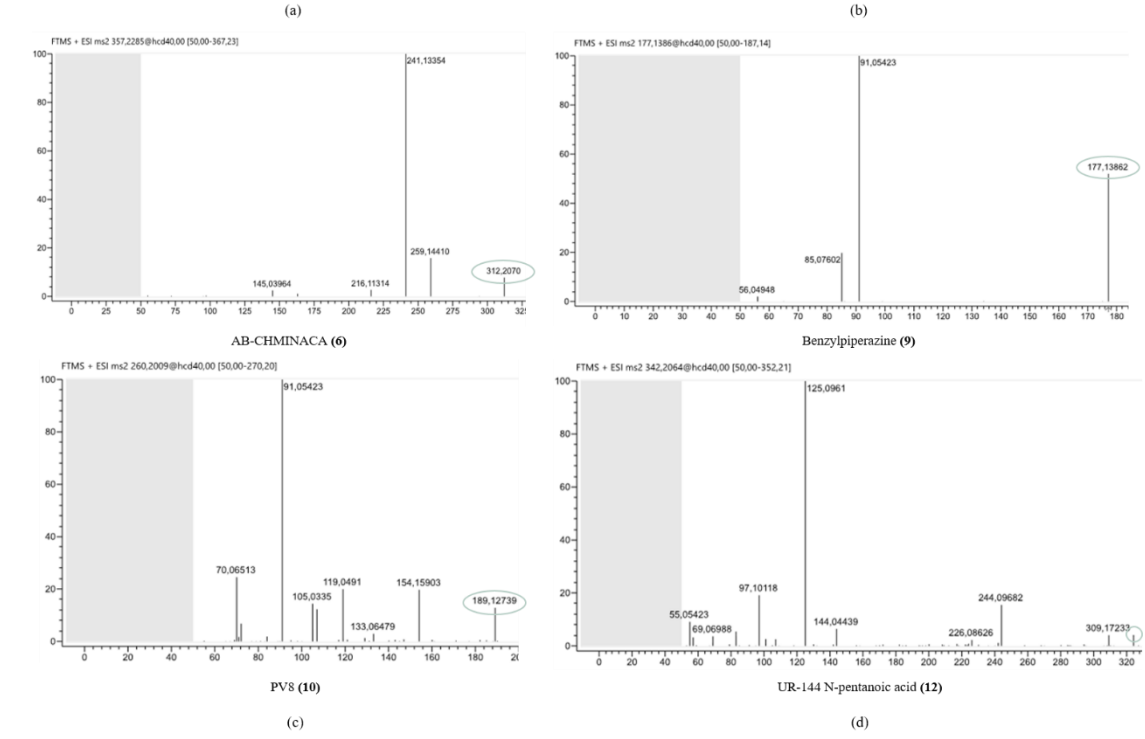


Figure 4.5 | (a) AB-CHMINACA, (b) benzylpiperazine, (c) PV8 and (d) UR-144 N-pentanoic acid: fragment ions (green circle) and their respective molecular formulae were obtained from mzCloud^{TM} spectra.

In some cases, JWH-073-M-2-OH-Ind (**7**) and benzylpiperazine (**9**) for example, the mass discrepancy was considerable. Regarding the remaining compounds, the variations were not substantial; however, the imprecise values contribute to inaccurate interpretations **(Table 4.1)**, therefore, it is fundamental to perform this step.

Table 4.1 | List of compounds missing the fragment molecular formulae. The blue colour represents the right values, whereas the dark red corresponds to the non-accurate ones.

Code	Compound	Drug class	F1mass (table on the right)	F2mass (table at the centre)	F3mass (table on the left)
1	Nitracaine	unknown	150.0186 (150.0185)	142.1589	-
2	Modafinil	unknown	152.0621		
3	MMMP	cathinones	165.0734 (165.0732)	88.0758 (88.0757)	128.1069 (128.1070)
4	Alfentanil	opioids	197.1285 (197.1284)	-	-
5	CUMYL-PeGA-CLONE	cannabinoids	255.1487 (255.1492)	-	-
6	AB-CHMINACA	cannabinoids	-	312.9159 (312.2070)	-
7	JWH-073-M-2-OH-Ind	cannabinoids	-	326.1539 (328.1332)	.
8	PB-22-N-pentanoic acid	cannabinoids	-	-	101.0898 (101.0597)
9	Benzylpiperazine	piperazine derivate	-	-	161.0818 (177.1386)
10	PV8	cathinones	-	-	189.1258 (189.1274)
11	AB-CHMINACA M1A	cannabinoids	-	-	239.1175 (239.1179)
12	UR-144 N-pentanoic acid	cannabinoids	-	-	324.1955 (324.1958)

In order to have an insight into the number of compounds that hold fragment ions, a second pivot table was used. This table comprised the InChIKeys, molecular formulae of the three most intense peaks and respective IDs. The following methodology was employed:

- Filter: F1MF, F2MF and F3MF (considering the ones without their molecular formulae)
- Row: InChIKey
- Values: Count of ID

We realised that roughly 21% (n=230) of the compounds on the database contained fragments, whereas 79% (n=865) were still missing their fragments **(Chart 4.1)**.

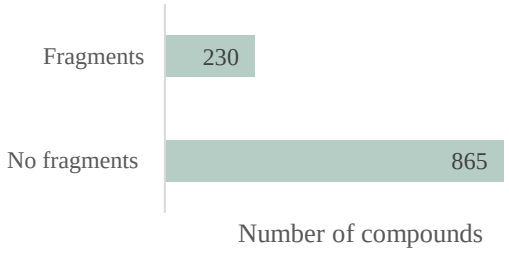


Chart 4.1 | Number of compounds containing fragments and no fragments in October 2018.

Approximately 61 systematic names (*i.e.* IUPAC names) of compounds on the database were also adjusted by using OPSIN and TiHKAL platforms. Of note, it is important to make sure that the substances correspond to the right IUPAC name, so that a valid InChIKey can be generated. The latter parameter is an identifier, characteristic of one specific chemical compound and allows its identification, which might be useful when searching for it on databases or websites. In addition, the compounds might be named in different manners as well as their systematic names can sometimes undergo a small number of variations, therefore the InChIKey is crucial for recognition of a substance.

Nevertheless, in some situations it was not possible to find the proper IUPAC name, since there was not any InChIKey associated, which makes the investigation challenging, especially when we are dealing with isomers. There were two examples of compounds where it was not feasible to obtain the proper structures and therefore, their InChIKeys: JWH-073-2-Methyl and JWH-073-3-Methyl. The question is: where is the methyl group located? Is in tail, on the linked group? According to the available information on the database, we know that both substances belong to the cannabinoids class and have the following fragment ions: *m/z* 214.1226 and 155.0491 **(Table 4.2)**.

Table 4.2 | Available information on the database for JWH-073-2-Methyl and JWH-073-3-Methyl.

Compound	RT	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	Drug class	LAB	Method
JWH-073-2-Methyl	12.31	$C_{24}H_{23}NO$	342.1852	$C_{11}H_7O$ *155.0491*	$C_{14}H_{16}NO$ *214.1226*	-	STD	CB	Uni. of Athens	BPS
JWH-073-3-Methyl	12.39	$C_{24}H_{23}NO$	342.1852	$C_{11}H_7O$ *155.0491*	$C_{14}H_{16}NO$ *214.1226*	-	STD	CB	Uni. of Athens	BPS

RT: Retention time, PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, CB: Cannabinoids, Uni. of Athens: University of Athens, BPS: Brukers Pesticide Screener.

In this way, the starting point would be searching for JWH-073 on the Internet, like some drug websites (*e.g.* TiHKAL), in order to get a general chemical structure and try to make slight modifications by adding the methyl group in a more likely place (*e.g.* the tail). The potential structures were drawn by using *ChemBioDraw Ultra 16.0* and the fragments were predicted by applying the fragmentation tools from the software **(Figure 4.6)**.

Based on the fragmentation patterns, the initial assumption might be right, and both methyl groups may be placed on the unit corresponding to the tail. Afterwards the IUPAC name of each structure was attained, as well as the respective InChIKey.

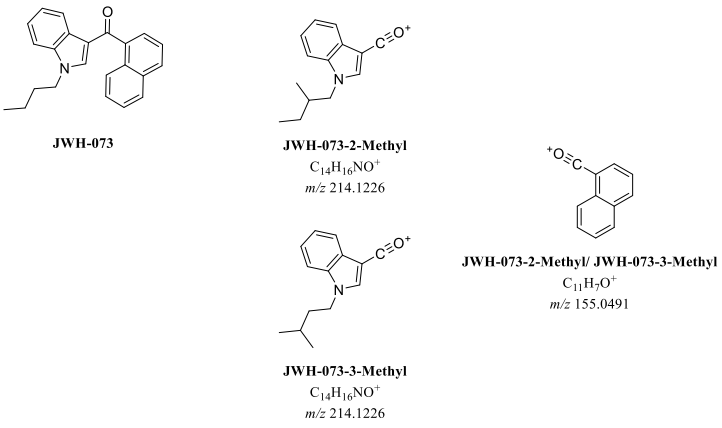


Figure 4.6 | Possible findings for JWH-073-2-Methyl and JWH-073-3-Methyl based on their fragmentation patterns (methyl group in the tail).

The possibility of having the methyl group on the linked group (*i.e.* naphthalene ring) was also tested. However, we realised that the fragment ions did not match with the ones acquired **(Table 4.3)**. Hence, we may conclude that the methyl group is positioned in the tail.

Table 4.3 | Fragment ions for JWH-073-2-Methyl and JWH-073-3-Methyl with methyl group on the naphthalene ring.

Compound	Fragment ion	
	tail + core + carbonyl group	linked group + carbonyl group (methyl group on the naphthalene ring)
JWH-073-2-Methyl JWH-073-3-Methyl	200.1070	169.0648

In case of plant-based substances, it was not possible to distinguish between two alkaloids such as, mitragyna alkaloid 1 and mitragyna alkaloid (*Paynantheine*), since both present the same molecular formula and thus, identical molecular mass. Although the third fragment ion is distinct, it is not easy to similar proceed to the previous example **(Table 4.4)**.

Table 4.4 | Available information on the database for mitragyna alkaloid 1 and mitragyna alkaloid (*Paynantheine*).

Compound	RT	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	Drug class	LAB	Method
Mitragyna alkaloid 1	7.54	$C_{23}H_{28}N_2O_4$	397.2122	$C_{11}H_{12}NO$ *174.0913*	$C_{12}H_{18}NO_3$ *224.1281*	$C_{13}H_{14}NO$ *200.1070*	STD	P&E	Uni. of Athens	BPS
Mitragyna alkaloid (*Paynantheine*)	6.44	$C_{23}H_{28}N_2O_4$	397.2122	$C_{11}H_{12}NO$ *174.0913*	$C_{12}H_{18}NO_3$ *224.1281*	$C_{13}H_{17}NO_3$ *235.1203*	STD	P&E	Uni. of Athens	BPS

RT: Retention time, PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, P&E: Plants & extracts, Uni. of Athens: University of Athens, BPS: Brukers Pesticide Screener.

To conclude the validation process, 333 entries from EMCDDA and RESPONSE libraries were removed, since they were not contributing with valuable information. In other words, entries comprising only the exact parent mass, molecular formula, systematic name, InChIKey and drug class (non-experimental data) were deleted. Although, this was applied when more than one entry for the same compound was present, otherwise the entry would be kept on the database until a user includes that specific substance. For example, 5-IT **(Table 4.5)** contained two additional entries encompassing the fragmentation ions **(first and second rows, Table 4.5)**. It is possible to verify that an identical compound can have a variety of names (*i.e.* synonymous); therefore, the InChIKey is a key parameter for ensuring a proper differentiation between substances.

Table 4.5 | Example of 5-IT and its two additional entries.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	Drug class	LAB	InChIKey
5-IT	$C_{11}H_{14}N_2$	175.1230				EMCDDA	I	CPH	AUL-GMISRJWGT-BA-UHFF-FAOYSA-N
5-(2-Aminopropyl)indole	$C_{11}H_{14}N_2$	175.1230	C_9H_8N *130.0651*	$C_{11}H_{12}N$ *158.0964*	C_8H_7N *117.0573*	STD	I	Uni. of Athens	
5-API	$C_{11}H_{14}N_2$	175.1230	C_9H_8N *130.0651*	C_8H_7N *117.0573*	$C_{11}H_{12}N$ *158.0964*	STD	I	Adelaide	

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, EMCDDA: European Monitoring Centre for Drugs and Drug Addiction, STD: Standard, I: Indolalkylamines, CPH: Copenhagen, Uni. of Athens: University of Athens.

After completing the validation process, Australian, English and Finnish entries were included on the database containing 193, 8 and 2 compounds, respectively.

The emerging number of fentanyl analogues and synthetic cannabinoids on drug market forced us to create two sub-libraries that will be described below: fentanyl analogues fragment prediction **(A)** and synthetic cannabinoids fragment prediction **(B)**, in order to understand the fragmentation patterns for both these drug classes. In this way, a list of compounds from each category was provided by the Section of Forensic Chemistry (Copenhagen, Denmark). The **Chart 4.2** illustrates the exact amount: 51 fentanyl analogues and 215 synthetic cannabinoids, in comparison with the available methods on HighResNPS database. The fragmentation study was conducted based on articles and at the last stage, mzCloud™ was used to assure that the theoretical prediction was on the right track, so that we could acquire the three most intense peaks. Before moving forward to the prediction, it is important to define the conditions on which the fragments ions were searched on mzCloud™, since the collision gas (*i.e.* argon) employed on Xevo G2-S QTOF is different from the Orbitrap used on mzCloud™ (*i.e.* nitrogen). Hence, the intensity of the peaks might suffer some variations. We have noticed that 40 eV is more likely to provide akin outcomes. However, there might be some cases where this voltage is not available on mzCloud™, thus 30 eV may be considered instead. We also need to make sure that we are looking at the correct ionization mode (*i.e.* ESI^+).

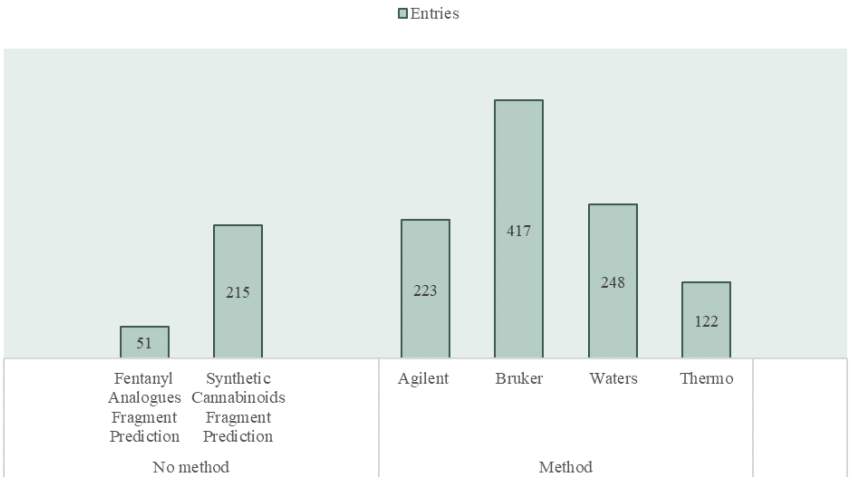


Chart 4.2 | Number of fentanyl analogues and synthetic cannabinoids (no method) used for fragment prediction, in comparison with the available entries on the database containing a method.

A. Fentanyl analogues fragment prediction

To carry out the fragmentation study for fentanyl analogues, a list of 51 substances were provided by the Section of Forensic Chemistry **(compounds 1-51, Appendix C)**. In order to give an insight concerning the fragmentation routes for this class of drugs, their characteristic ions were determined based on their chemical structures. The common cleavages [143,144] are described below **(Figure 4.7)** and a supplemental material comprising the theoretical fragment ions for each compound can be found on **Appendix D**.

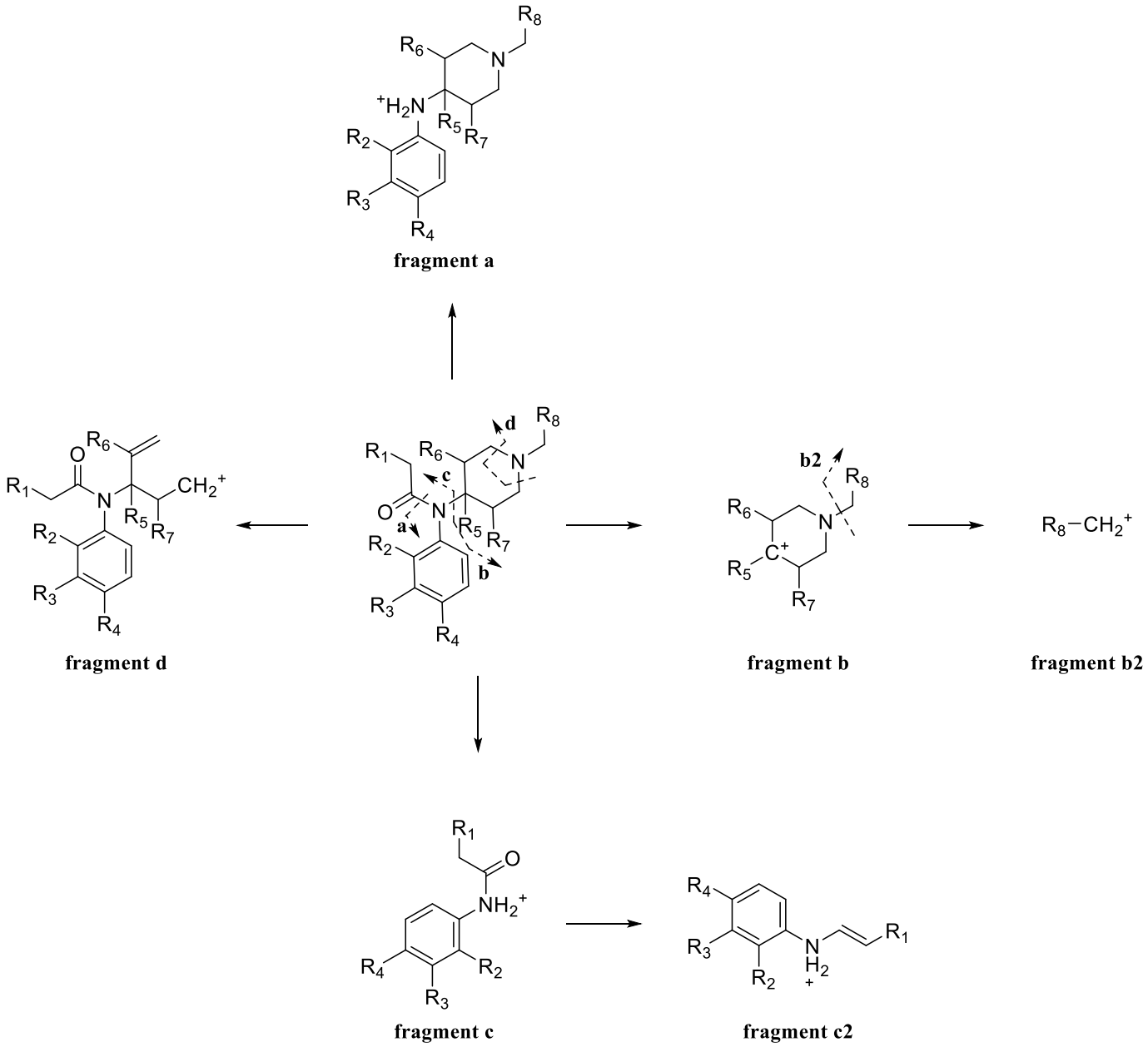


Figure 4.7 | Proposed fragmentation pathways for fentanyl analogues (Appendix D).

Fragment a **(Figure 4.7 and Appendix D)**

This fragment corresponds to the degradation of the amide functional group through the breakdown of the nitrogen and carbonyl carbon bond. The phenomenon is observed for a certain number of compounds such as, 4-fluorobutyrfentanyl **(10)** with a *m/z* value 299.1918 ($C_{19}H_{24}FN_2^+$), β-hydroxythiofentanyl **(23)** with a *m/z* value 285.1420 ($C_{17}H_{21}N_2S^+$), butyryl fentanyl **(25)** and isobutyryl fentanyl **(38)** with a *m/z* value 281.2012 ($C_{19}H_{25}N_2^+$).

Fragment b **(Figure 4.7 and Appendix D)**

This fragment is the result of breaking the amide moiety bond and the piperidine ring. In case, the fentanyl analogues do not hold any substituent in the phenethyl group, it is possible to observe the formation of a

carbocation with a *m/z* value 188.1434 ($C_{13}H_{18}N^+$). The obtained fragment may be subjected to a consecutive disruption between the alpha-carbon and the piperidine ring, giving rise to *m/z* 105.0699 ($C_8H_9^+$, **fragment b2**). Both fragments are detected in the following fentanyl analogues: 2-fluoro acrylfentanyl **(1)**, 2-fluorobutyryl fentanyl **(2)**, 2-fluoroisobutyryl fentanyl **(3)**, 2-methoxy butyryl fentanyl **(4)**, 3-fluoro methoxyacetyl fentanyl **(5)**, 3-fluorofentanyl **(6)**, 3-fluoroisobutyryl fentanyl **(7)**, 4-fluoro acrylfentanyl **(9)**, 4-fluorofentanyl **(11)**, acrylfentanyl **(16)**, benzodioxole fentanyl **(20)**, cyclobutyl fentanyl **(31)**, cyclohexyl fentanyl **(32)**, cyclopropyl fentanyl **(33)**, despropionyl 3-fluorofentanyl **(34)**, despropionyl *p*-fluoro fentanyl **(35)**, FIBF **(36)**, methoxyacetyl fentanyl **(40)**, *m*-fluorofentanyl **(42)** and ocfentanil **(46)**. The previous values may suffer slight modifications depending on the type of moiety attached to the piperidine ring. In this sense, we have noticed that:

1. butyryl norfentanyl **(26)**, isobutyryl norfentanyl **(39)**, methoxyacetyl norfentanyl **(41)** and norfentanyl **(45)** lead to a product ion with *m/z* 84.0808 ($C_5H_{10}N^+$), which comprehend a hydrogen linked to the piperidine ring, instead of a phenethyl group;
2. benzylfentanyl **(22)** results in a fragment ion with *m/z* 174.1277 ($C_{12}H_{16}N^+$) that is characteristic of substances comprising a benzyl group;
3. thiofentanyl **(50)** originates a fragment with *m/z* 194.0998 ($C_{11}H_{16}NS^+$), which consists of replacing a phenethyl for a thienylethyl group;
4. 3-methylfentanyl **(8)**, 4-methyl fentanyl **(13)**, α-methyl butyryl fentanyl **(17)**, α-methylacetylfentanyl **(19)**, β-methylfentanyl **(24)**, *cis*-3-methyl butyryl fentanyl **(28)** and *cis*-mefentanyl **(30)** generate an ion with *m/z* 202.1590 ($C_{14}H_{20}N^+$), which contain a methyl group on the piperidine ring (for **6**,**28** and **30**), at the alpha-carbon position (for **17** and **19**), in the phenyl group (for **13**, **28** and **30**) or at the beta-carbon position (for **24**);
5. α-methyl thiofentanyl **(18)** and *cis*-3-methylthiofentanyl **(29)** produce a fragment with *m/z* 208.1154 ($C_{12}H_{18}NS^+$) that present a methyl group at the alpha-carbon position or on the piperidine ring and a thienylethyl group linked to it, respectively;
6. ohmefentanyl **(47)** gives rise to a product ion with *m/z* 218.1539 ($C_{14}H_{20}NO^+$) that corresponds to a methyl group on the piperidine ring and a hydroxyl group at the beta-carbon position;
7. sufentanil **(49)** leads to a fragment with *m/z* 238.1260 ($C_{13}H_{20}NOS^+$), which is related to a methoxymethyl group on the piperidine ring and a thienylethyl group attached to it;
8. carfentanil **(27)** promotes the formation of an ion with *m/z* 246.1489 ($C_{15}H_{20}NO_2^+$), this one involves the presence of a carboxylate group on the piperidine ring and the phenethyl group linked to it.

Regarding the **fragment b2 (Figure 4.7 and Appendix D)**, it is important to underline that compounds containing a thienylethyl group attached to the piperidine ring are more likely to induce the generation of an ion with *m/z* 111.0263 ($C_6H_7S^+$). In addition, some product ions with *m/z* 202.1590 may undergo a subsequent fragmentation leading to a minor fragment ion (*m/z* 119.0855, $C_9H_{11}^+$). Moreover, substances holding a benzyl group linked to the piperidine ring may result in a carbocation with *m/z* 91.0542 ($C_7H_7^+$).

Fragment c **(Figure 4.7 and Appendix D)**

This fragment is produced due to the prior fragment. In other words, the product ion losses a carbonyl oxygen and therefore, it experiences dehydration, which brings about the fragment termed **c2**. The fragment c is observed for butyryl norfentanyl **(26)** and isobutyryl norfentanyl **(39)** with a *m/z* value 164.1070 ($C_{10}H_{14}NO^+$), acetyl norfentanyl **(15)** with a *m/z* value 136.0757 ($C_8H_{10}NO^+$), methoxyacetyl norfentanyl **(41)** with a *m/z* value 166.0863 ($C_9H_{12}NO_2^+$) and norfentanyl **(45)** with a *m/z* value 150.0913 ($C_9H_{12}NO^+$). A further fragmentation generates the following *m/z* values: 341.1682 ($C_{20}H_{25}N_2OS^+$) for β-hydroxythiofentanyl **(23)** and 134.0964 ($C_9H_{12}N^+$) for ohmefentanyl **(47)**, which correspond to **fragment c2**, as mentioned previously.

Fragment d **(Figure 4.7 and Appendix D)**

This fragment involves the degradation of the piperidine ring owing to the cleavage on both C(2)-N and C(6)-N, which promotes the formation of specific *m/z* values relying on possible substituents on *N*-benzene group and carbonyl of the amide functional group such as, 232.1132 ($C_{14}H_{15}FNO^+$) for 2-fluoro acrylfentanyl **(1)**, 250.1238 ($C_{14}H_{17}FNO_2^+$) for 3-fluoro methoxyacetyl fentanyl **(5)**, 248.1445 ($C_{15}H_{19}FNO^+$) for 4-fluoro-butyrfentanyl **(10)**, 260.1645 ($C_{16}H_{22}NO_2^+$) for 4-methoxy-butyryl fentanyl **(12)**, 202.1226 ($C_{13}H_{16}NO^+$) for acetyl norfentanyl **(15)**, 228.1383 ($C_{15}H_{18}NO^+$) for cyclopropyl fentanyl **(33)**, 230.1539 ($C_{15}H_{20}NO^+$) for iso-butyryl fentanyl **(38)** and 244.1696 ($C_{16}H_{22}NO^+$) for valeryl fentanyl **(51)**.

In order to make sure, that the proposed fragmentation might be applied to future analogues, three compounds from the fentanyls list were selected without any criteria and thereafter, compared with the entries available on HighResNPS. In this way, acetyl norfentanyl **(15)**, FIBF **(36)** and valeryl fentanyl **(51)** were analysed. For the first example **(compound 15, Table 4.6)**, it is observed that the first product ions match between the three users, regarding the second one is not possible to define the right value; we would need the data from Aarhus to ensure a proper interpretation and the third fragment does not allow us to get any further details regarding the fragmentation. Although the peak intensity is not similar, it presents an identical value to one of the second fragments. Thus, we may conclude that they agree at least in two fragments.

Table 4.6 | First example: acetyl norfentanyl.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
Acetyl norfentanyl	$C_{13}H_{18}N_2O$	219.1492	$C_5H_{10}N$ ***84.0808***	$C_{13}H_{16}NO$ ***202.1226***	$C_8H_{10}NO$ ***136.0757***	TRT	CPH	FAFP
			$C_5H_{10}N$ ***84.0808***	$C_8H_{10}NO$ ***136.0757***	-	STD	CPH	WFTS
			$C_5H_{10}N$ ***84.0808***	-	-	STD	Aarhus	Bruker 3

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, TRT: Theoretical, STD: Standard, CPH: Copenhagen, FAFP: Fentanyl Analogues Fragment Prediction, WFTS: Waters Forensic Toxicology Screening.

Concerning the second example **(compound 36, Table 4.7)**, the first fragments fit with all laboratories. In terms of the second fragments, two out of one are consistent. Nevertheless, the third one does not provide any additional information.

Table 4.7 | Second example: FIBF.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
FIBF	$C_{23}H_{29}FN_2O$	369.2337	$C_{13}H_{18}N$ ***188.1434***	C_8H_9 ***105.0699***	$C_{15}H_{19}FNO$ ***248.1445***	STD	CPH	WFTS
			$C_{13}H_{18}N$ ***188.1434***	-	-	Seizure	Aarhus	Bruker 3
			$C_{13}H_{18}N$ ***188.1434***	C_8H_9 ***105.0699***	-	TRT	CPH	FAFP
			$C_{13}H_{18}N$ ***188.1434***	$C_{19}H_{24}FN_2$ ***299.1918***	-	LIB	CPH	TF QExactive 1

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, TRT: Theoretical, LIB: Library, CPH: Copenhagen, WFTS: Waters Forensic Toxicology Screening, FAFP: Fentanyl Analogues Fragment Prediction.

For the last example **(compound 51, Table 4.8)**, the first and third fragments are compatible, while the second ones generate dissimilar values.

Table 4.8 | Third example: valeryl fentanyl.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
Valeryl fentanyl	$C_{24}H_{32}N_2O$	365.2587	$C_{13}H_{18}N$ ***188.1434***	$C_{19}H_{25}N_2$ ***281.2012***	$C_{16}H_{22}NO$ ***244.1696***	TRT	CPH	FAFP
			$C_{13}H_{18}N$ ***188.1434***	C_8H_9 ***105.0699***	$C_{16}H_{22}NO$ ***244.1696***	STD	CPH	WFTS

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, TRT: Theoretical, STD: Standard, CPH: Copenhagen, FAFP: Fentanyl Analogues Fragment Prediction, WFTS: Waters Forensic Toxicology Screening.

Based on this study, we might deduce that the predicted fragmentation may assist the analyst in interpreting most of the generated product ions within the fentanyls drug class, giving an insight into an array of substances. In addition, the first fragments coincide always with the data reported by other laboratories and, to a lesser extent, second and third fragment ions. Hence, the fragmentation pathways can be applied to future analogues, since it provides an overview concerning the type of fentanyls that we are dealing with. Moreover, it is possible to notice that according to their chemical structure, they may adopt a similar fragmentation pattern, which is especially useful when analysing novel compounds, since they are characterised by minor modifications in their skeleton.

B. Synthetic cannabinoids fragment prediction

To conduct the fragmentation study for synthetic cannabinoids, a list containing 215 compounds were culled from the Section of Forensic Chemistry **(compounds 1-215, Appendix E)**. In order to give a comprehensive overview regarding the fragmentation patterns among this class of drugs, they were divided into subgroups according to their chemical structures, as suggested by *K. Sekuła et al.: Analysis of Fragmentation Pathways of New-Type Synthetic Cannabinoids Using Electrospray Ionization* [44]:

- **B.1** SCs with adamantly ring
- **B.2** SCs with naphthalene or quinoline ring
- **B.3** SCs with 2,2,3,3-tetramethylcyclopropyl (TMCP) moiety
- **B.4** PINACA-SCs
- **B.5** FUBINACA-SCs
- **B.6** CHMINACA-SCs
- **B.7** Other SCs

B.1 SCs with adamantly ring **(Appendix F)**

Compounds holding an adamantly ring as the linked group were more likely to give rise to an ion with a theoretical *m/z* value of 135.1168, which corresponds to the most intense peak in the MS/MS spectrum (mzCloud™), thus it is considered a characteristic ion for synthetic cannabinoids containing this type of ring. In addition, ions with the following *m/z* values: 98.0964 and 112.1121 (for AM1248 **(73)**), 214.1226 (for JWH 018 adamantyl carboxamide **(112)**), 243.1128 (for ATHPINACA isomer 2 **(85)**) and 284.1645 (for SER-601 (**192)**) were also observed. The previous fragments were associated with the core of SCs combined with a linker and a tail section. However, for AM1248 **(73)** the fragment ions corresponded merely to the tail section. Of note, these SCs may consist of a pair of aryl groups attached to a carbonyl or carboxyamide function and the core might be either an indole (R_1, R_3 = -CH) or an indazole (R_1, R_3 = -N) moiety. For both cases, the fragmentation pathways are illustrated in **Figure 4.8**. It is important to highlight that SER-601 **(192)** contains a 4-hydroxyquinoline on its core instead, nevertheless the fragmentation pattern is identical since it comprises an adamantly ring and a carboxyamide linker.

From a chemical point of view, the groups bonded to the carboxyamide and carbonyl functions can be easily broken. In this specific situation, the dominant ion corresponds to the **fragments a** and **c**, respectively **(Figure 4.8)**. The remaining part of the molecule is not always visible in the MS/MS spectrum. Moreover, the *m/z* values may differ according to the substituents R_1, R_2, R_3 and R_4, as well as the core considered (*e.g.* indole and indazole).

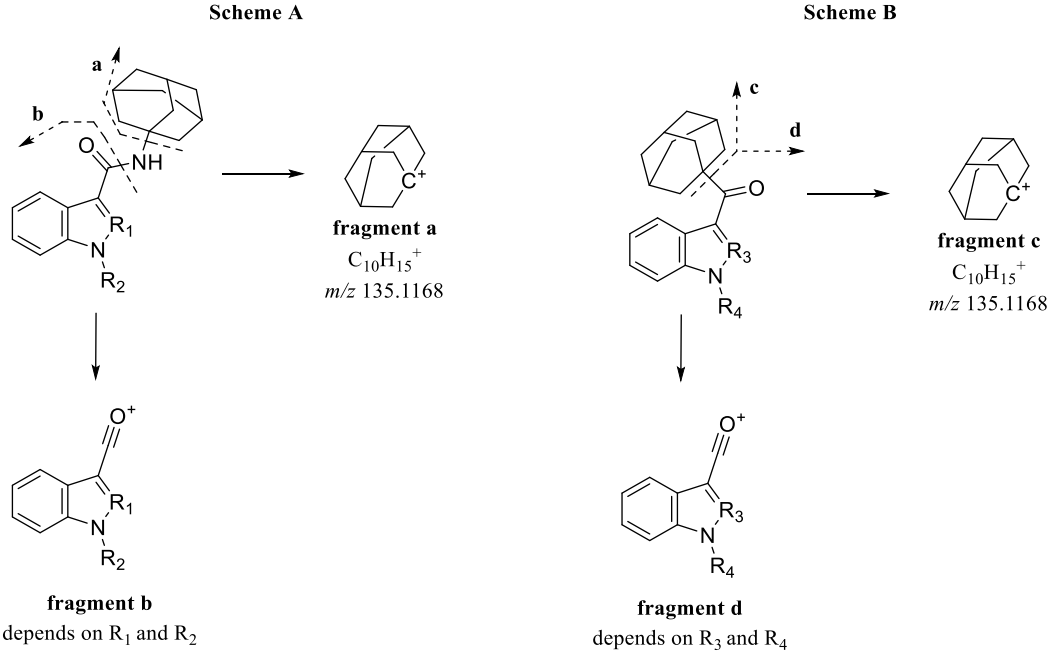


Figure 4.8 | Fragmentation pathways for SCs with adamantly ring: carboxyamide function (scheme A) and carbonyl function scheme B) (Appendix F).

B.2 SCs with naphthalene or quinoline ring

This sub-group consists of compounds containing three possible linker groups *i.e.* carbonyl, carboxyamide and carboxyl moiety, which will be mentioned in more detail below. In this way, the linker will determine the characteristic fragment ions obtained.

- with carbonyl group **(Appendix G)**

These SCs contain a naphthalene ring combined with a core section, which can be either an indole or indazole via a carbonyl group. It is worth noting that, other types of cores might be observed, however they are less frequent than the aforementioned moieties. All the analysed compounds comprised a naphthalene ring instead of a quinoline as the linked group. The fragmentation pattern is defined by the following rational: the oxygen bounded to the carbon atom (*i.e.* linker) and the nitrogen present in the indole/indazole ring might be the primary sites of protonation [145]. Nevertheless, the oxygen atom is more basic compared to the nitrogen atom, since the latter one is doubly conjugated with an aromatic ring. In this way, the C-C bonds adjacent to the oxygen atom are easily cleaved, which may produce two typical product ions [145], denoted as **fragment a** and **fragment b** in **Figure 4.9**. The first **fragment a** coincided to naphthoyl cation. If the naphthalene ring is unsubstituted, the generated ions will correspond to 155.0491 ($C_{11}H_7O^+$, **fragment a**) and the **fragment b** may lead to a determined value that relies on R_2 R_3 and R_4. In other words, for the latter fragment its values will be dictated by the chemical composition of each substituent. It is important to remember that the *m/z* values will be different according to the core.

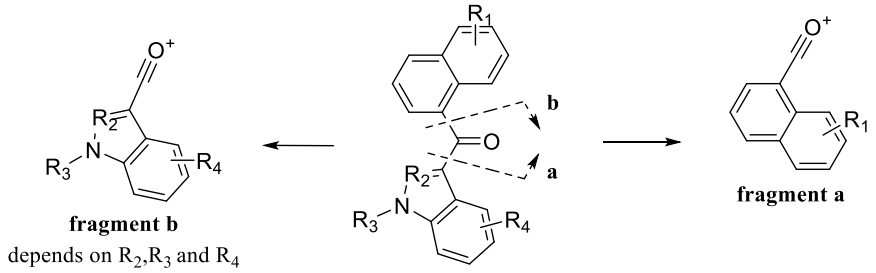


Figure 4.9 | Proposed fragmentation pathways for SCs containing a naphthalene ring and a carbonyl group (Appendix G).

Some examples of the investigated compounds were used to elucidate the common fragment ions for two specific cores moieties:

1. indole (R_2 = -CH) **(Appendix G)**

Formation of characteristic ions of the indole ring: *m/z* 144.0444 (for 1-naphthoyl indole **(1)**), 189.0295 and 277.0983 (for AM1235 **(71)**), 232.1132 (for Cl2201 **(90)**), 109.0448 and 252.0819 (for FUB-JWH 018 **(103)**), 242.1539 (for JWH 020 **(117)**), 212.1070 (for JWH 022 **(118)**), 172.0757 (for JWH 071 **(119))**, among others.

2. indazole (R_2 = -N) **(Appendix G)**

Formation of characteristic ions of the indazole ring: *m/z* 145.0396 and 213.1022 (*e.g.* 5-bromo THJ 018 **(9)**).

As mentioned previously, there are some compounds that do not contain any of the prior cores such as, EG-018 **(95)**. Nonetheless, the applied fragmentation rules can still be employed. In this sense, the fragment ion corresponding to the linker and core section (*i.e.* a carbazole) will be 264.1383 **(fragment b)**.

On the other hand, if the naphthalene ring contains substituents, the product ion corresponding to **fragment a** will differ. Thus, the presence of an alkyl group such as, a methyl, ethyl, propyl will give rise to 169.0648 (*e.g.* JWH 073 4-methylnaphthyl analog **(123)**), 183.0804 (*e.g.* JWH-210 **(148)**) and 197.0961 (*e.g.* JWH-180

(144)), respectively. In case of replacement of a hydrogen for a methoxy group, the following ion is formed: 185.0597 (*e.g.* JWH 080 **(127)**). In addition, a bromo, chlorine or fluorine substituent is also evident and the characteristic ions for both halogens are registered at *m/z* 232.9597 (*e.g.* JWH 387 **(133)**), 189.0102 (*e.g.* JWH-398 **(150)**) and 173.0397 (JWH 412 **(134)**), respectively.

- with carboxyl group **(Appendix H)**

These SCs comprises a naphthalene or quinoline ring combined with a core section, which can be either an indole or indazole moiety via a carboxyl group. The investigated compounds contained almost an equal number of naphthalene (R_1 = -CH) and quinolone (R_1 = -N) rings as part of the linked group. In terms of fragmentation, it was not observed a product ion (*i.e.* **fragment a**) that could correspond to the rings. As far as we know, any article has reported an explanation concerning this phenomenon. In this way, for all the analysed substances, the MS/MS spectra on mzCloud™ have shown no records associated to the carboxyl cleavage, which allowed to prove that the information above was valid. Based on this, the fragment ions were predicted without considering the prior bond break **(Figure 4.10)**. Therefore, we should focus merely on the **fragment b**, which may present an array of characteristic ions depending on the chemical structure of the substituents. It is important to notice that two main core sections can appear such as, indole (R_2 = -CH) and indazole (R_2 = -N) **(Figure 4.10)**. Hence, some of the following product ions may be generated: *m/z* 144.0444 and 232.1132 (for 5-fluoro PB-22 **(35)** and NM-2201 **(177)**), 144.0444 and 214.1226 (for CBL-018 **(88)**), lastly 97.1012, 144.0444 and 240.1383 (for QUCHIC **(187)**) for indole. On the other hand, 213.1022 and 251.1190 (for 5-fluoro NPB-22 **(34)**) and, 145.0396, 215.1179 and 233.1285 (for SDB-005 **(191)**) for indazole. Furthermore, four of the aforementioned compounds contained a naphthalene ring, CBL-018 **(88)**, NM-2201 **(177)**, QUCHIC **(187)**, SDB-005 **(191)**, whereas the remaining two a quinolone ring, 5-fluoro NPB-22 **(34)** and 5-fluoro PB-22 **(35)**.

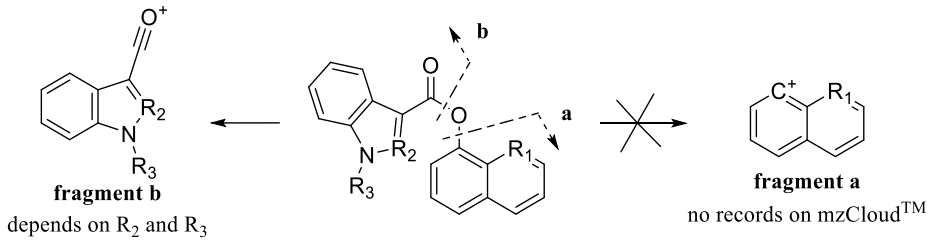


Figure 4.10 | Proposed fragmentation pathways for SCs containing a naphthalene/quinoline ring and a carboxyl group (Appendix H).

- with carboxyamide group **(Appendix I)**

These SCs differ in the linking group *i.e.* carboxyamide. Likewise in the previous two-linker groups, these compounds may consist of a core section, which can be either an indole or indazole as well as a linked group that will be a naphthalene (R_1 = -CH) or quinolone ring (R_1 = -N). In light of above, the **fragment a** is not registered in MS/MS spectra on mzCloud™, not even on research articles regarding the topic **(Figure 4.11)**. Thus, the **fragment b** will be the only considered. However, it is important to highlight that the *m/z* values will suffer some variations due to the chemical composition of the substituents R_2 and R_3. The following characteristic ions can be obtained: *m/z* 144.0444 and 214.1226 (for JWH 018 8-quinolinyl carboxamide **(110)** and NNEI **(178)**) for an indole ring, whereas *m/z* 145.0396 (for NNEI 2-indazole isomer **(179)**), 145.0396 and 215.1179 (for MN-18 **(175)**) and 213.1022 and 233.1085 (for 5-fluor MN-018 **(32)** and 5-fluor THJ **(40)**) for an indazole ring. From the prior compounds, only two contained a quinoline ring, 5-fluor THJ **(40)** and JWH 018 8-quinolinyl carboxamide **(110)**, all the others presented a naphthalene ring.

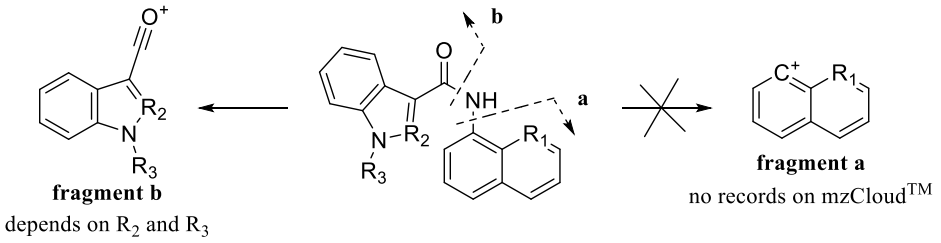


Figure 4.11 | Proposed fragmentation pathways for SCs containing a naphthalene/quinoline ring and a carboxyamide group (Appendix I).

B.3 SCs with 2,2,3,3-tetramethylcyclopropyl (TMCP) moiety **(Appendix J)**

Compounds comprising a TMCP moiety as part of the linked group are more likely to generate a fragment ion with a *m/z* value 125.0961 **(fragment a)**, which corresponds to the cleavage of the bond adjacent to the carbonyl function. It is also verified a product ion associated with the TMCP only (*m/z* 97.1012, **fragment b**). Depending on the core and tail section chemical composition, it is possible to obtain different *m/z* values such as, 257.1285 (for A-796260 **(44)**), 242.1176 (for A-834735 **(46)**), 242.1414 (for AB-005 **(49)**), 233.1085 (for FAB-144 **(99)**), 252.0819 (for FUB-144 **(102)**), 214.1226 (for UR-144 **(198)**), 244.0968 (for UR-144 5-pentanoic acid metabolite **(199)**), 248.0837 (for UR-144 *N*-(2-chloropentyl) analog **(201)**), 292.0332 (for UR-144 *N*-(5-bromopentyl) analog **(204)**), 242.1539 (for UR-144 *N*-(5-methylhexyl analog **(206)** and UR-144 *N*-heptyl analog **(207)**), 232.1132 (for XLR-11 **(211)**), 248-1081 (for XLR-11 *N*-(4-hydroxypentyl) metabolite **(213)**), 212.1070 (for XLR-11 *N*-(4-pentenyl) analog **(214)**) and 254.0787 (for XLR-12 **(215)**), which correspond to **fragment c**, whereas 187.0900 (for A-836339 **(48)**) is related to **fragment d**, as illustrated in **Figure 4.12**. In addition, some of the aforementioned substances can still suffer fragmentation, giving rise to the following *m/z* values: 114.0913 (for A-834735 **(46)**), 112.1121 (for AB-005 **(49)**), 213.1022 (for FAB-144 **(99)**), 109.0448 (for FUB-144 **(102)**) and 55.0542 (for azidoindolene 1 **(86)**) is the only associated to **fragment e**. The first ions are formed by the disruption of the bond between the TMCP moiety and carbonyl group, whereas the second ones represent a subsequent fragmentation of the previous product ions, which occurs in the tail section. It is commonly observed the removal of a fluorine atom at the end of the tail and the bond breaking of a fluorobenzyl (*e.g.* **compound 102**) or morpholinoethyl group (*e.g.* **compound 44**) from the core section.

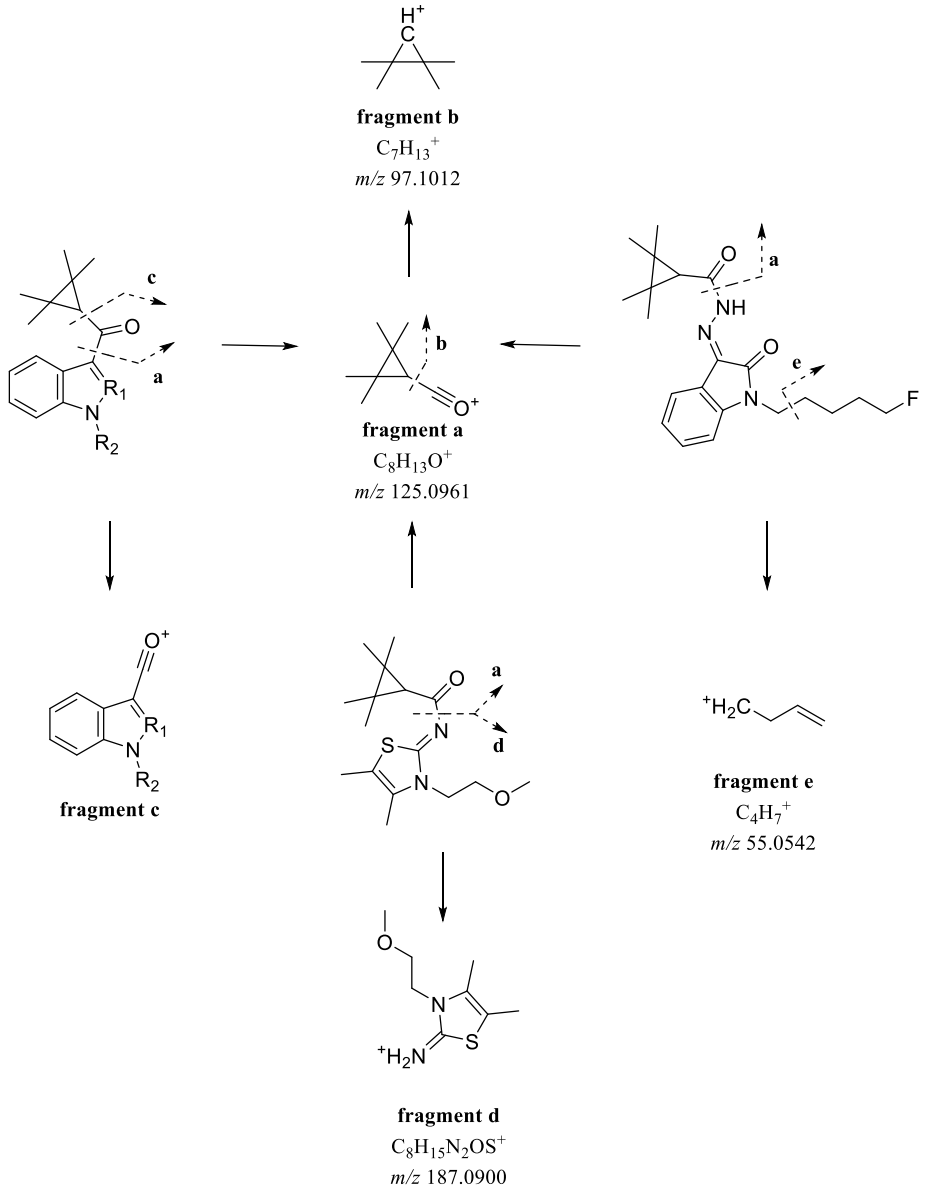


Figure 4.12 | Proposed fragmentation pathways for SCs with a TMCP moiety (Appendix J).

B.4 PINACA-SCs **(Appendix K)**

These SCs are characterized by an indazole ring with a pentyl chain and a carboxamide linker. For these compounds the following main ions might be generated **(Figure 4.13)**: **fragment a** corresponds to the loss of a NH_2 group. All the substances containing an amide moiety may result in *m/z* 332.1769 (for 5-fluoro AB-PINACA **(14)**), *m/z* 346.1925 (for 5-fluoro ADB-PINACA **(18)**, 5-fluoro ADB-PINACA isomer 2 **(19)** and 5-fluoro-2-ADB-PINACA isomer 2 **(41)**) and *m/z* 328.2020 (for ADB-PINACA **(67)**). while *m/z* 213.1022 and *m/z* 233.1085 are associated to **fragments b** and **c,** respectively. Both are registered for 5-fluoro AB-PINACA **(14)**, 5-fluoro ADB-PINACA **(18)** and 5-fluoro ADB-PINACA isomer 2 **(19)**. Of note, the first value **(fragment b)** is associated with the loss of a fluorine atom and the second one **(fragment c)** is derived from the C-N cleavage of the amide group. In addition, when the pentyl chain does not comprise a halogen (R_3) at the end of the chain, it is possible to observe a *m/z* value of 215.1179 for ADB-PINACA **(67)** and CUMYL-PINACA

(92). In case of substances containing a CUMYL moiety (5F-CUMYL-PINACA **(12)** and CUMYL-PINACA **(92)**), their linked group (*i.e.* isopropylbenzene/cumyl) differ from the previous ones. Therefore, the *m/z* values will be: 119.0855 and 91.0542 **(fragment a)**. The latter fragment ion is formed by the cleavage between the isopropyl and benzene.

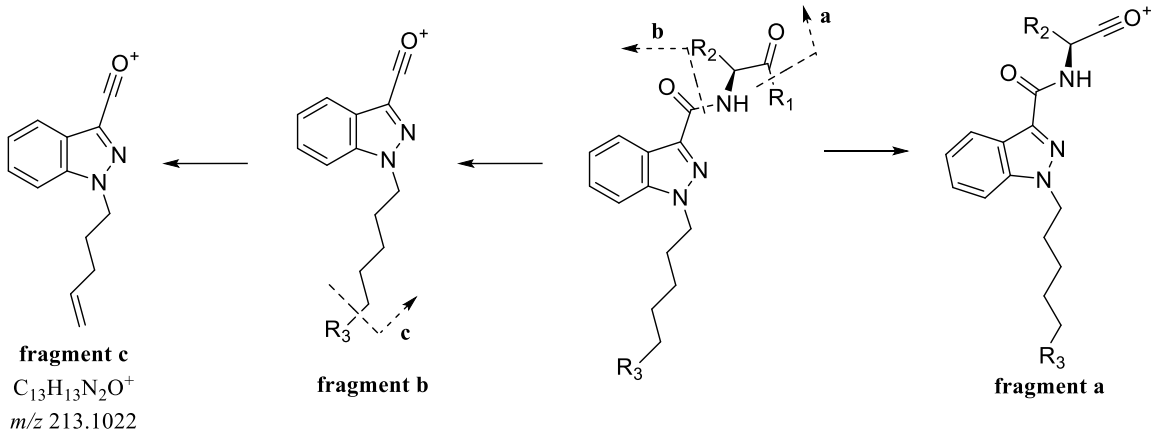


Figure 4.13 | Proposed fragmentation pathways for PINACA compounds (Appendix K).

B.5 FUBINACA-SCs **(Appendix L)**

The FUBINACA sub-group is composed by a fluorobenzyl group that makes part of the tail section, an indazole ring and carboxamide linker. These compounds exhibited a common fragmentation pattern **(Figure 4.14)**. It was observed that the C-N cleavage of the amide group would result in **fragment b** with a *m/z* value 253.0772. Further fragmentation of this ion led to the formation of 109.0448, which is attributed to the dissociation of the bond between the core (*i.e.* indazole ring) and the fluorobenzyl group. These product ions were registered for ADB-FUBINACA **(65)**, APP-FUBINACA **(83)**, EMB-FUBINACA **(97)**, MDMB-FUBINACA **(167)** and MMB-FUBINACA **(174)**. For three compounds, it was possible to notice the cleavage of the bond between the carbonyl group and the carbon attached to R_2, which formed the following ions: *m/z* 352.1456 (for EMB-FUBINACA **(97)**), *m/z* 338.1663 (for MDMB-FUBINACA) and *m/z* 324.1507 (for MMB-FUBINACA **(174)**). This fragment corresponds to the loss of an ester group. In addition, compounds comprising an amide moiety are more likely to loss a NH_2 group giving rise to **fragment a**. The mass values will vary according to the chemical composition of substituent R_2: *m/z* 366.1612 (for ADB-FUBINACA **(65)**) and *m/z* 400.1456 (for APP-FUBINACA **(83)**).

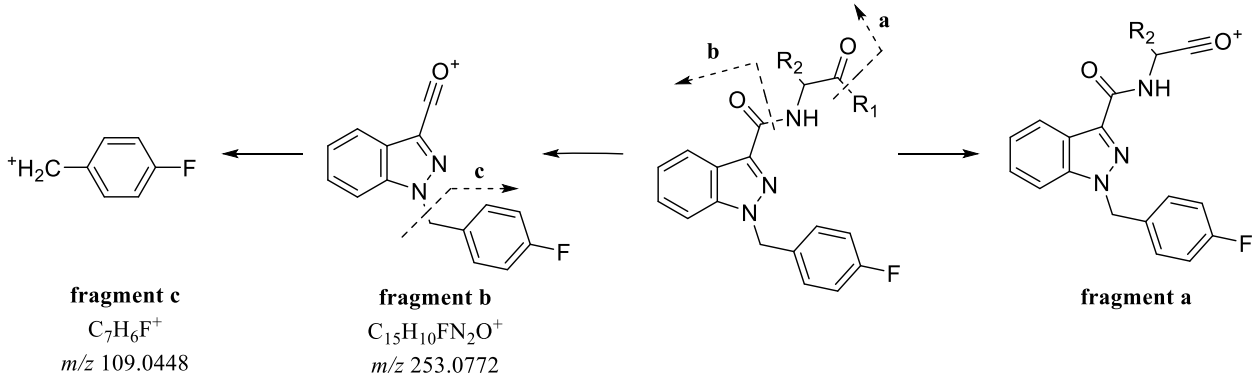


Figure 4.14 | Proposed fragmentation pathways for FUBINACA compounds (Appendix L).

B.6 CHMINACA-SCs **(Appendix M)**

These type of SCs consist of a cyclohexylmethyl group in the tail section, an indazole as a core and a carboxamide linker. For CHMINACA, it is possible to distinguish two characteristic ions with *m/z* values 241.1335 **(fragment b)** and 145.0396 **(fragment c)**, both illustrated in **Figure 4.15**. The first one corresponds to the C-N cleavage of the amide group, which was detected for the following compounds: AB-CHMINACA **(52)**, AB-CHMINACA metabolite M2 **(56)**, AB-CHMINACA metabolite M6 **(59)**, APP-CHMINACA **(82)**, MAB-CHMINACA **(153)**, MAB-CHMINACA metabolite M10 **(155)**, MA-CHMINACA **(160)**, MDMB-CHMINACA **(166)** and MO-CHMINACA **(176)**. In other words, this fragment is derived from the core (*i.e.* indazole) combined with the carbonyl group and cyclohexylmethyl moiety. A subsequent fragmentation led to the **fragment c**, which is related to the dissociation of the cyclohexylmethyl moiety from the core of the molecule. In addition, some substances have shown the dissociation of bond between the carbonyl group and the carbon attached to R_1 giving rise to **fragment a** with *m/z* values: 340.2020 (for AB-CHMINACA **(52)** and AB-CHMINACA 2-indazole isomer **(53)**), 388.2020 (for APP-CHMINACA **(82)**) 354.2227 (for MAB-CHMINACA **(153)**) and 370.2125 (for MAB-CHMINACA metabolite M1 **(154)**), which depend on R_1 substituents. In the case of methoxy groups, the product ions generated might be: *m/z* 312.2070 (for MA-CHMINACA **(160)**) and *m/z* 323.2227 (for MDMB-CHMINACA **(166)**).

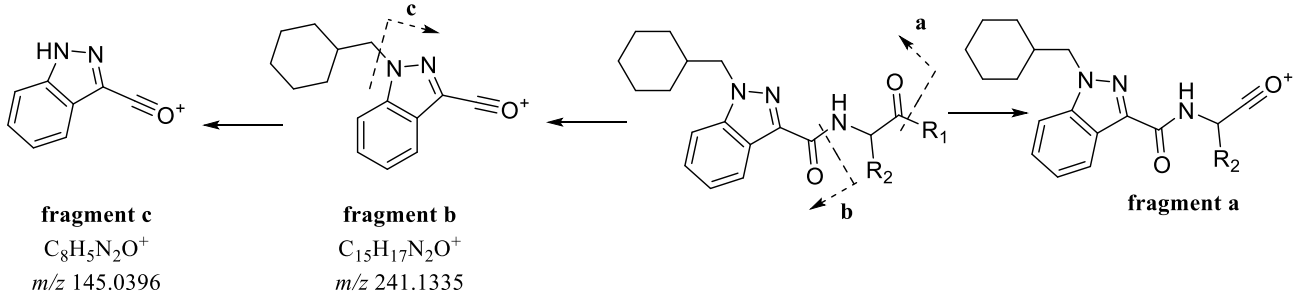


Figure 4.15 | Proposed fragmentation pathways for CHMINACA compounds. (Appendix M).

B.7 Other SCs

Some compounds on the list do not fit into any of the aforementioned sub-groups. However, no matter their chemical structure they may present similar fragmentation patterns, which will be briefly described below. Three substances were used to elucidate the fragmentation: 5-fluoro PY-PICA **(37)**, ADBICA **(66)** and MDMB-CHMICA **(165)**. In order to simplify the analysis, their structures and respective fragmentation pathways are depicted in **Figure 4.16**. In **Figure 4.161a**, it is possible to observe that the bonds adjacent to the carbonyl group will be cleaved giving rise to two main ions with *m/z* values: 98.0600 (**fragment a**), which corresponds to the linked group and linker and 232.1132 (**fragment b**) that is related to the linker, core and tail section. The latter one undergoes further fragmentation leading to **fragment c**, which is the result of dissociation of the tail section from the core with subsequent elimination of a fluorine atom. The **fragment b** is common to some SCs containing a naphthalene ring such as, 5-fluoro PB-22 (**(35), Appendix H)**, Cl2201 (**(90), Appendix G)** and NM-2201 ((**177), Appendix H)**.

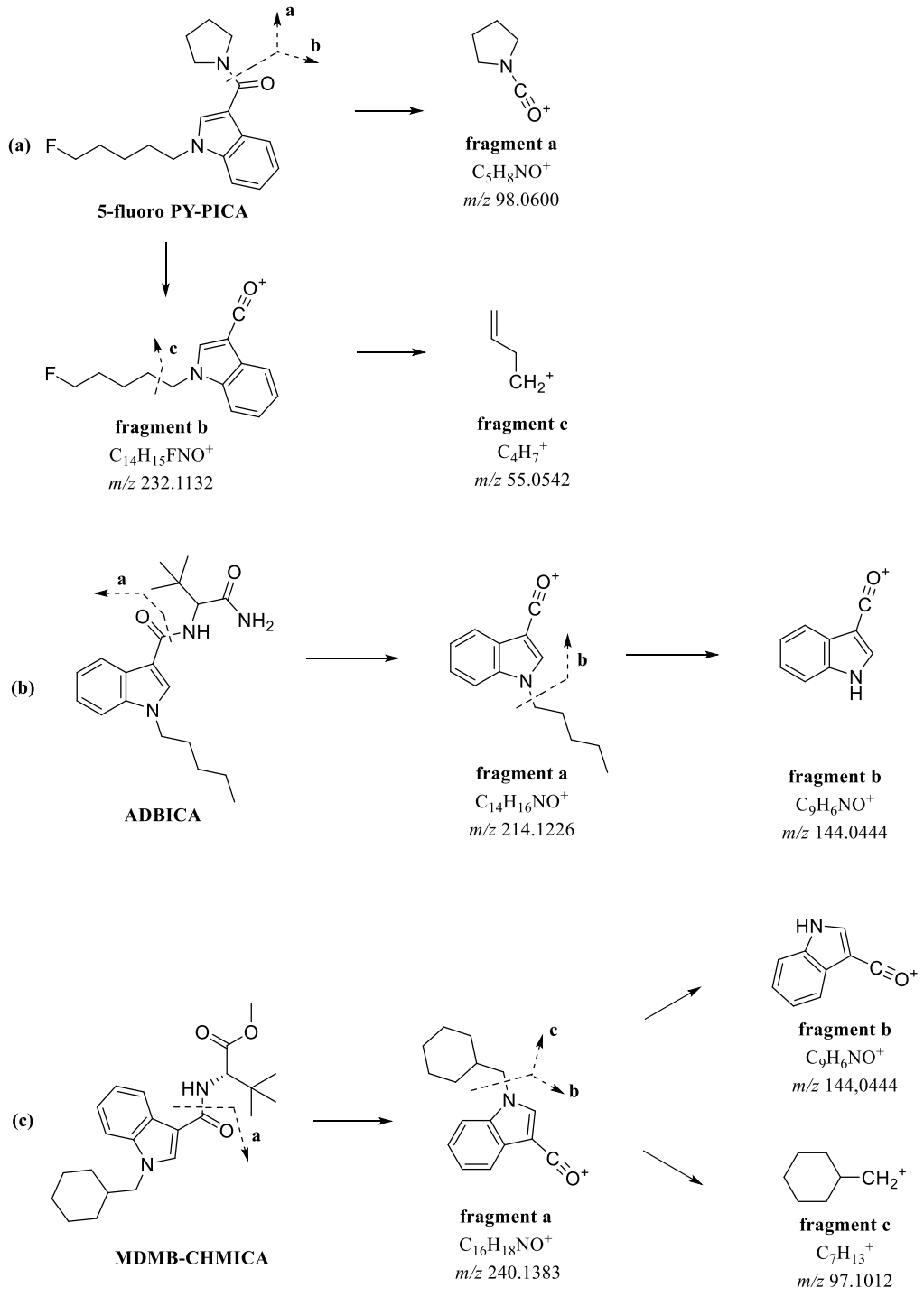


Figure 4.16 | Proposed fragmentation pathways for three compounds that do not fit into any of the aforementioned sub-groups: (a) 5-fluoro PY-PICA, (b) ADBICA and (c) MDMB-CHMICA.

For ADBICA **(Figure 4.16b)**, it is noticed that the bond between the C and N atoms is more likely to break forming an ion with *m/z* value 214.1226 (**fragment a**). This ion might also be fragmented generating the **fragment b** (144.0444), which is due to the cleavage of the bond between the core and the tail section. Regarding the last example **(Figure 4.16c)**, the C-N cleavage of the amide group is observed and thus, the **fragment a** is formed. Afterwards, the disintegration of the core from the tail section is detected. In this way, two ions may be obtained 144.0444 and 97.1012 that correspond to **fragments b** and **c**, respectively. The *m/z* value 144.0444

is shared by an array of compounds from the list such as, 1-naphthoyl indole (**(1), Appendix G)**, 5-fluoro PB-22 (**(35), Appendix H)** and NM-2201 (**(177), Appendix H)**. In addition, 214.1226 is also prevalent in other substances like, CBL-018 (**(88), Appendix H)** and JWH 018 8-quinolinyl carboxamide (**(110), Appendix I)**.

In order to investigate the effectiveness of the described SCs fragmentation, one compound belonging to each of the aforementioned sub-groups was selected from the HighResNPS database and its fragment ions were further compared to the fragmentation available from other laboratories. We started with SCs containing an adamantly group. For this case, the substance JWH 018 adamantyl carboxamide **(112)** was elected. On the database, there was only one entry from Labor Krone (DE) **(Table 4.9)**. It is possible to conclude that the German laboratory agrees with the first two fragments and includes a third product ion (*i.e.* 144.0444), which was not observed for the method SCFP. However, the predicted fragmentation matched well (*i.e.* two fragments out of three).

Table 4.9 | Example for SCs containing an adamantly group: JWH 018 adamantly carboxamide.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
JWH 018 adamantly carboxamide	$C_{24}H_{32}N_2O$	365.2587	$C_{10}H_{15}$ ***135.2268***	$C_{14}H_{16}NO$ ***214.1226***	C_9H_6NO ***144.0444***	STD	Labor Krone	WFTS
			$C_{10}H_{15}$ ***135.2268***	$C_{14}H_{16}NO$ ***214.1226***	-	TRT	CPH	SCFP

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, TRT: Theoretical, CPH: Copenhagen, WFTS: Waters Forensic Toxicology Screening, SCFP: Synthetic Cannabinoid Fragment Prediction.

For the second sub-group, a compound comprising a naphthalene ring was chosen (*e.g.* JWH-210 **(148)**). In **Table 4.10**, it is illustrated its fragment ions reported in two more methods (BPS and Thermo 2). In comparison with the theoretical fragmentation (*i.e.* method SCFP), the product ions were compatible (*i.e.* two fragments out of two).

Table 4.10 | Example for SCs comprising a naphthalene ring: JWH-210.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
JWH-210	$C_{26}H_{27}NO$	370.2165	$C_{13}H_{11}O$ ***183.0804***	$C_{14}H_{16}NO$ ***214.1226***	-	TRT	CPH	SCFP
			$C_{13}H_{11}O$ ***183.0804***	$C_{14}H_{16}NO$ ***214.1226***	-	STD	Uni. of Athens	BPS
			$C_{13}H_{11}O$ ***183.0804***	$C_{14}H_{16}NO$ ***214.1226***	-	STD	CPH	Thermo 2

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, TRT: Theoretical, STD: Standard, CPH: Copenhagen, Uni. of Athens: University of Athens, SCFP: Synthetic Cannabinoid Fragment Prediction, BPS: Brukers Pesticide Screener.

In case of SCs with a TMCP moiety, UR-144 **(198)** was considered **(Table 4.11)**. It was verified that all the laboratories agreed in the first fragment and in general, they assented to the second product ion, except the Labor Krone. Regarding the last fragment, there was no coherence between users. Nevertheless, we may conclude that at least two ions coincide and thus, they corresponded to the predicted fragmentation (*i.e.* method SCFP).

Table 4.11 | Example of SCs with a TMCP moiety: UR-144.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
UR-144	$C_{21}H_{29}NO$	312.2322	$C_8H_{13}O$ ***125.0961***	C_7H_{13} ***97.1012***	$C_{14}H_{16}NO$ ***214.1226***	STD	Labor Krone	WFTS
			$C_8H_{13}O$ ***125.0961***	$C_{14}H_{16}NO$ ***214.1226***	-	STD	Ade-laide	Agilent 1
			$C_8H_{13}O$ ***125.0961***	$C_{14}H_{16}NO$ ***214.1226***	-	STD	Uni. of Athens	BPS
			$C_8H_{13}O$ ***125.0961***	$C_{14}H_{16}NO$ ***214.1226***	C_9H_6NO ***144.0444***	STD	RCMP-NFLS	WFTS
			$C_8H_{13}O$ ***125.0961***	$C_{14}H_{16}NO$ ***214.1226***	C_7H_{13} ***97.1012***	TRT	CPH	SCFP

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, TRT: Theoretical, Uni. of Athens: University of Athens, CPH: Copenhagen, WFTS: Waters Forensic Toxicology Screening, BPS: Brukers Pesticide Screener SCFP: Synthetic Cannabinoid Fragment Prediction.

5F-CUMYL-PINACA **(12)**, which makes part of substances with a PINACA acronym, shared the first fragment with Adelaide; however, Aarhus did not consider the same fragment ion. In addition, the second product ion did not suffer any modification. In relation to the last one, there was not an agreement between the laboratories **(Table 4.12)**.

Table 4.12 | Example from PINACA sub-group: 5F-CUMYL-PINACA.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
5F-CUMYL-PINACA	$C_{22}H_{26}FN_3O$	368.2132	$C_{13}H_{14}FN_2O$ ***233.1085***	C_9H_{11} ***119.0855***	$C_{13}H_{17}FN_3O$ ***250.1350***	STD	Adelaide	Agilent 1
			$C_{13}H_{14}FN_2O$ ***233.1085***	C_9H_{11} ***119.0855***	$C_{13}H_{13}N_2O$ ***213.1022***	TRT	CPH	SCFP
			$C_{13}H_{17}FN_3O$ ***250.1350***	-	-	R	Aarhus	Bruker 3

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, TRT: Theoretical, R: RESPONSE, CPH: Copenhagen, SCFP: Synthetic Cannabinoid Fragment Prediction.

Concerning the FUBINACA acronym, ADB-FUBINACA **(65)** was the selected compound. It was noticed that the fragment ions were completely in concordance with the theoretical fragmentation **(Table 4.13)**. Although, it was not observed a third product ion.

Table 4.13 | Example from FUBINACA sub-group: ADB-FUBINACA.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
ADB-FUBI-NACA	$C_{21}H_{23}FN_4O_2$	383.1878	$C_{15}H_{10}FN_2O$ ***253.0772***	C_7H_6F ***109.0448***	-	TRT	CPH	SCFP
			$C_{15}H_{10}FN_2O$ ***253.0772***	C_7H_6F ***109.0448***	$C_{20}H_{21}FN_3O$ ***338.1663***	STD	Uni. of Athens	WFTS
			$C_{15}H_{10}FN_2O$ ***253.0772***	C_7H_6F ***109.0448***	$C_{20}H_{21}FN_3O$ ***338.1663***	STD	Labor Krone	Thermo 2

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, TRT: Theoretical, STD: Standard, CPH: Copenhagen, Uni. of Athens: University of Athens, SCFP: Synthetic Cannabinoid Fragment Prediction, WFTS: Waters Forensic Toxicology Screening.

For the CHMINACA acronym, AB-CHMINACA **(52)** subjected to the fragmentation analysis. Most of the obtained fragment ions were conformed to the predicted fragmentation, especially the first and second ions. Even though, the Labor Krone has considered a different intensity order. Some laboratories suggested the presence of more than two fragments **(Table 4.14)**.

Table 4.14 | Example from CHMINACA sub-group: AB-CHMINACA.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
AB-CHMINACA	$C_{20}H_{28}N_4O_2$	357.2285	$C_{15}H_{17}N_2O$ ***241.1335***	$C_{19}H_{26}N_3O$ ***312.2070***	-	STD	Ade-laide	Agilent 1
			$C_{15}H_{17}N_2O$ ***241.1335***	$C_{19}H_{26}N_3O$ ***312.2070***	-	TRT	CPH	SCFP
			$C_{15}H_{17}N_2O$ ***241.1335***	$C_{19}H_{26}N_3O$ ***312.2070***	$C_8H_5N_2O$ ***145.0396***	STD	Uni. of Athens	BPS
			$C_{15}H_{17}N_2O$ ***241.1335***	$C_8H_5N_2O$ ***145.0396***	$C_{19}H_{26}N_3O$ ***312.2070***	STD	Labor Krone	WFTS
			$C_{15}H_{17}N_2O$ ***241.1335***	$C_{19}H_{26}N_3O$ ***312.2070***	-	R	Aarhus	Bruker 3

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, STD: Standard, TRT: Theoretical, R: RESPONSE, CPH: Copenhagen, Uni. of Athens: University of Athens, SCFP: Synthetic Cannabinoid Fragment Prediction, BPS: Brukers Pesticide Screener, WFTS: Waters Forensic Toxicology Screening.

Lastly but not least, substances that do not fit into any of the prior sub-groups such as, 5-fluoro PY-PICA **(37)**. In this case, the first two product ions accorded between each other, but this did not apply to the third fragment, the values were divergent **(Table 4.15)**.

Table 4.15 | Example from other SCs: 5-fluoro PY-PICA.

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***	Origin	LAB	Method
5-fluoro PY-PICA	$C_{18}H_{23}FN_2O$	303.1867	$C_{14}H_{15}FNO$ ***232.1132***	C_5H_8NO ***98.0600***	C_4H_7 ***55.0542***	TRT	CPH	SCFP
			$C_{14}H_{15}FNO$ ***232.1132***	C_5H_8NO ***98.0600***	C_9H_6NO ***144.0444***	R	CPH	WFTS
			$C_{14}H_{15}FNO$ ***232.1132***	C_5H_8NO ***98.0600***	-	R	Aarhus	Bruker 3

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions, TRT: Theoretical, R: RESPONSE, CPH: Copenhagen, SCFP: Synthetic Cannabinoid Fragment Prediction, WFTS: Waters Forensic Toxicology Screening.

Based on this analysis, it might be inferred that the predicted fragmentation could be useful in determining and interpreting future analogues. The established strategy provides the reader with comprehensive analytical information by exploring a variety of chemical structures for synthetic cannabinoids. However, this approach is not always perfect, therefore in some occasions it will not be possible to define a proper methodology, since the core skeleton might be slightly different. In other words, the inclusion of novel core sections, halogens in the tail sections, modification of functional groups in the linkers as well as linked groups may contribute to a completely distinct structure. According to the examples given for each sub-group, it is possible to verify that the first and second product ions match between laboratories, however this does not always apply to the third one, which commonly presents odd values. Nevertheless, we could conclude that the first ion might be of paramount importance for distinguishing the type of SCs and the remaining fragment ions conduce to additional information concerning the chemical composition of a certain compound within this drug class. In addition, it is worth noting that the ion values generated from the *N*-hetero rings may suggest the kind of core (*i.e.* indole or indazole). In this way, odd values result in substances comprising an indazole, whereas even ones originate substances containing an indole [146]. In light of above, the strategy is not always feasible due to chemical modifications that may take place. JWH-175 **(Figure 1.3b)**, which belongs to SCs with a naphthalene ring, is an example of this, the replacement of the usual linkers by a methylene group does not comply with the prior fragmentation principle.

At this stage, the database comprised 1758 entries **(Figure 4.17a)**. Owing to the number of multiple entries for the same substance, the previous estimative was considered with reference to compounds available on the database in March 2019. After downloading the database into an excel file, it was possible to notice the presence of 1251 compounds: 9 aminoindanes, 25 arylalkylamines, 20 arylcyclohexylamines, 41 benzodiazepines, 433 cannabinoids, 158 cathinones, 77 indolalkylamines, 131 opioids, 176 phenethylamines, 28 piperazine derivates, 27 piperidines & pyrrolidines, 20 plants & extracts and 106 unknown **(Figure 4.17b)**. In comparison with the initial data **(Figure 4.2)**, it might be concluded that the increase of entries is not considerable; however, we should take into account the quantity of compounds present on the database instead. Thus, 156 novel substances were additional included, contributing with significant theoretical information, more especifically in terms of fragment ions.

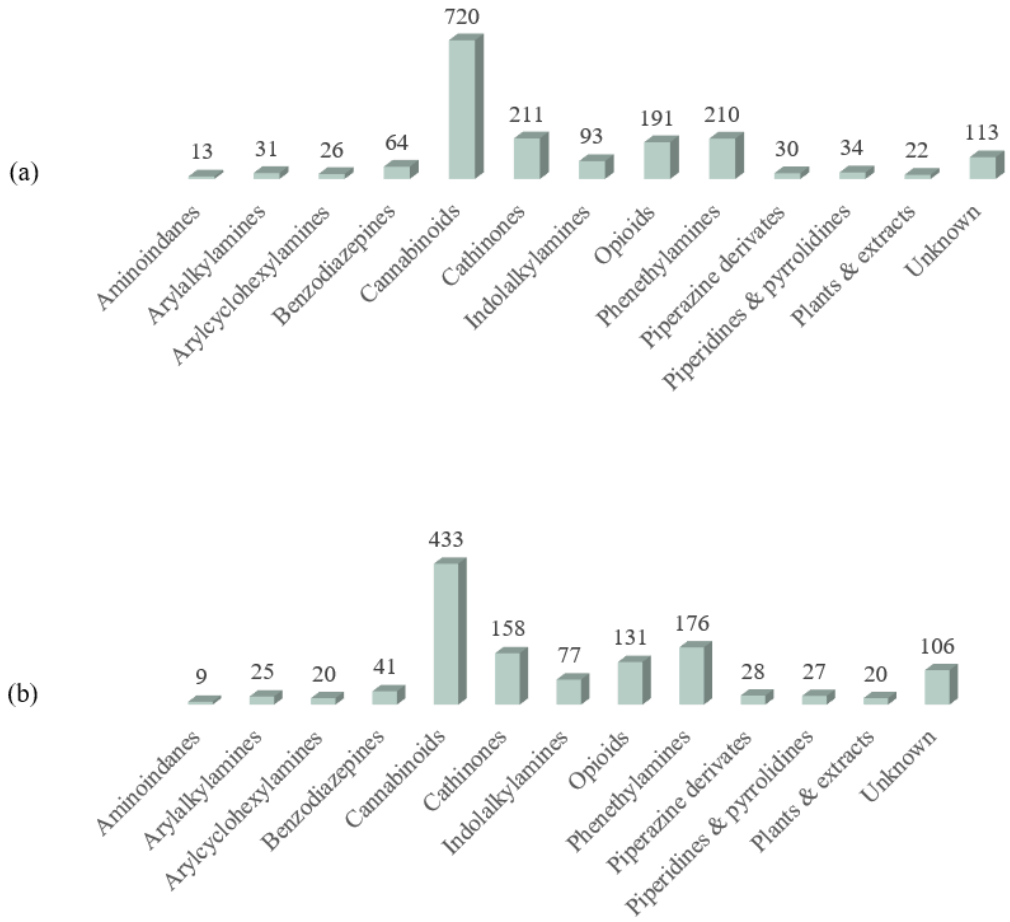


Figure 4.17 | HighResNPS database in March 2019: (a) number of entries and (b) number of compounds per drug class.

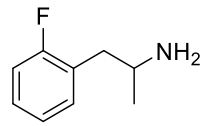
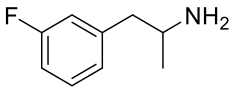
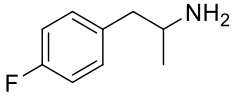
According to the reported data, we have witnessed an evolution of the database concerning fragmentation. At that point, 775 NPS contained fragment ions while 476 were still absent. However, it is important to understand how well these fragments match between laboratories/users. In this way, with the support of pivot tables this study was carry out. We realised that:

- 273 compounds comprising fragments were reported by more than one laboratory. Of this, 99.6% (n=272) shared at least one fragment;
- from 1501 fragments, 1004 were distinct fragments recorded by 273 compounds. 60% (n=603) of them were from more than one laboratory.

Throughout the database, it is possible to notice the presence of 349 isomer groups. In other words, compounds comprising an identical molecular formula, nevertheless a distinct chemical structure. Of note, positional isomers (*i.e. ortho-*, *meta-* and *para-*) are the more evident, which are characterised by different groups at the same carbon skeleton. For these cases, it might be challenging to identify the correct structure due to similarities in their fragment ions. The following examples: 2-fluoroamphetamine/3-fluoroamphetamine/4-fluoroamphetamine **(Table 4.16)** and 2-APB/4-APB/5-APB/6-APB **(Table 4.17)**, represent some NPS that cannot be easily distinguished. The three product ions available on the database for the previous substances are not enough to contribute for a proper interpretation, which means that we are not able to determine the

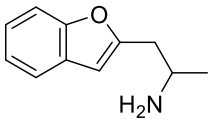
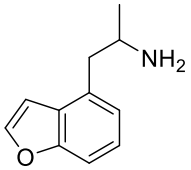
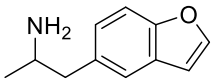
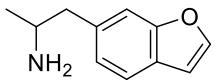
position of any of the substituents, since they can be present in more than one place in the considered molecules. For the given examples, we have noticed in fact, that they comprehend equal values for the fragment ions. Therefore, it might be difficult to define the position of the substituents, since the fragmentation pattern is identical.

Table 4.16 | Example 1: 2-fluoroamphetamine, 3-fluoroamphetamine and 4-fluoroamphetamine (phenethylamines).

 2-fluoroamphetamine		 3-fluoroamphetamine		 4-fluoroamphetamine	
Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***
2-fluoroamphetamine	$C_9H_{12}FN$	154.1026	C_7H_6F ***109.0448***	$C_9H_{10}F$ ***137.0761***	C_5H_4F ***83.0292***
3-fluoroamphetamine	$C_9H_{12}FN$	154.1026	C_7H_6F ***109.0448***	$C_9H_{10}F$ ***137.0761***	C_5H_4F ***83.0292***
4-fluoroamphetamine	$C_9H_{12}FN$	154.1026	C_7H_6F ***109.0448***	$C_9H_{10}F$ ***137.0761***	C_5H_4F ***83.0292***

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions.

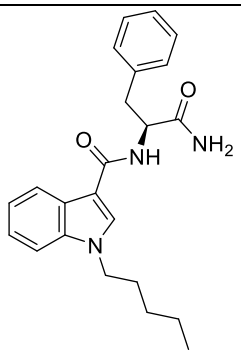
Table 4.17 | Example 2: 2-APB, 4-APB, 5-APB and 6-APB (arylalkylamines).

 2-APB		 4-APB		 5-APB	 6-APB
Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***
2-APB	$C_{11}H_{13}NO$	176.1070	-	-	-
4-APB	$C_{11}H_{13}NO$	176.1070	-	-	-
5-APB	$C_{11}H_{13}NO$	176.1070	C_9H_7O ***131.0491***	$C_{11}H_{11}O$ ***159.0804***	C_7H_7 ***91.0542***
6-APB	$C_{11}H_{13}NO$	176.1070	C_9H_7O ***131.0491***	$C_{11}H_{11}O$ ***159.0804***	C_7H_7 ***91.0542***

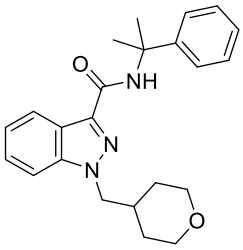
PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions.

On the other hand, it is important to highlight that there are also compounds on the database that share the exact same mass, nonetheless it is possible to use the fragments to differentiate them, *e.g.* APP-PICA and CUMYL-THPINACA both synthetic cannabinoids with a parent mass of 378.2176 Da **(Table 4.18)**. The first substance contains an indole (*i.e.* core), whereas the second one an indazole and the two comprise a carboxyamide linker attached to distinct moieties. In this way, the product ions allow us to distinguish these isomers, since the values associated to each of them are different.

Table 4.18 | APP-PICA and CUMYL-THPINACA: the exact same mass but different fragment ions.



APP-PICA



CUMYL-THPINACA

Compound	PMF	Pmass	F1MF ***F1mass***	F2MF ***F2mass***	F3MF ***F3mass***
APP-PICA	$C_{23}H_{27}N_3O_2$	378.2176	$C_{14}H_{16}NO$ ***214.1226***	C_9H_6NO ***144.0444***	-
CUMYL-THPINACA	$C_{23}H_{27}N_3O_2$	378.2176	$C_{14}H_{15}N_2O_2$ ***243.1128***	C_9H_{11} ***119.0855***	$C_6H_{11}O$ ***99.0804***

PMF: Parent Molecular Formula, Pmass: Parent mass, F1MF/F2MF/F3MF: Molecular formula of the three most abundant fragment ions, F1mass/F2mass/F3mass: Mass of the three most abundant fragment ions.

After completing all these steps, the HighResNPS database can be further converted into an in-house library (*i.e.* a UNIFI library). To perform this process:

1. HighResNPS is exported as a spreadsheet (excel file);
2. The available entries are renamed to [name@item number], in order to enable the identification based on analytical information submitted by multiple laboratories. Thereof, the entries are further generated into .mol files, which depend on their IUPAC names.

This procedure requires progamming skills, hence to facilitate the entire process it will be possible to find the required files on the database, allowing an easy access to the prior data to any user. In this way, they will be able to operate HighResNPS in their own LC-HRMS systems. However, at the moment the available files are exclusive to the UNIFI software. In this sense, the second part of this thesis will scrutinize a different approach to interpret the data without the need to import the database to the MS software.

4.2 Part II – Screening for NPS in seizures

In order to test the applicability of HighResNPS database in forensic analysis, 36 interesting samples **(see appendix N)** confiscated by the Danish authorities from 2015 to 2019 were processed using UHPLC-QTOF-MS. However, only 14 samples will be described below in more detail, since the remaining ones resulted in identical outcomes or were not considered NPS, instead they made part of the so-called traditional drugs (*e.g.* heroin and MDMA). The mass spectral data for these 14 unknown compounds was acquired in positive mode and in data-independent acquisition (DIA). Of note, this acquisition mode enables the analysis of all precursor ions in the ion source, thereof are subjected to fragmentation, which is induced by a ramp of collision energy (CE) from 10 to 40 eV.

In light of above, the screening approach was based on the use of HighResNPS as an online mass spectral database, without the need to import the library. In order to carry out this study, a workflow composed by four steps was established:

1. The total ion chromatogram (TIC): focus on the more intense peak;
2. Precursor ion $(M+H)^+$: search this value (*i.e.* at least select one decimal) on HighResNPS database to narrow down the possible candidates;
3. Fragmentation profile: examine the fragments, comparing the experimental values with the ones available on HighResNPS. In case this is not possible, resort to mzCloud™ to obtain supplemental information at this level. If none of these methods provide enough data, the reference standards might be purchased and/or nuclear magnetic resonance (NMR) spectroscopy techniques (especially for positional isomers) should be performed;
4. Structure elucidation: a possible chemical structure of the compound is determined.

4.2.1 Sample 1

The **Figure 4.18a** illustrates a dominant chromatographic peak at 3.36 min. The presence of a sodium adduct $(M+Na)^+$ at *m/z* 230.1146 demonstrates that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 208.1332 ($C_{21}H_{17}NO_2$) with a mass error (*i.e.* the difference between the experimental mass and the theoretical mass) of 0.0 mDa.

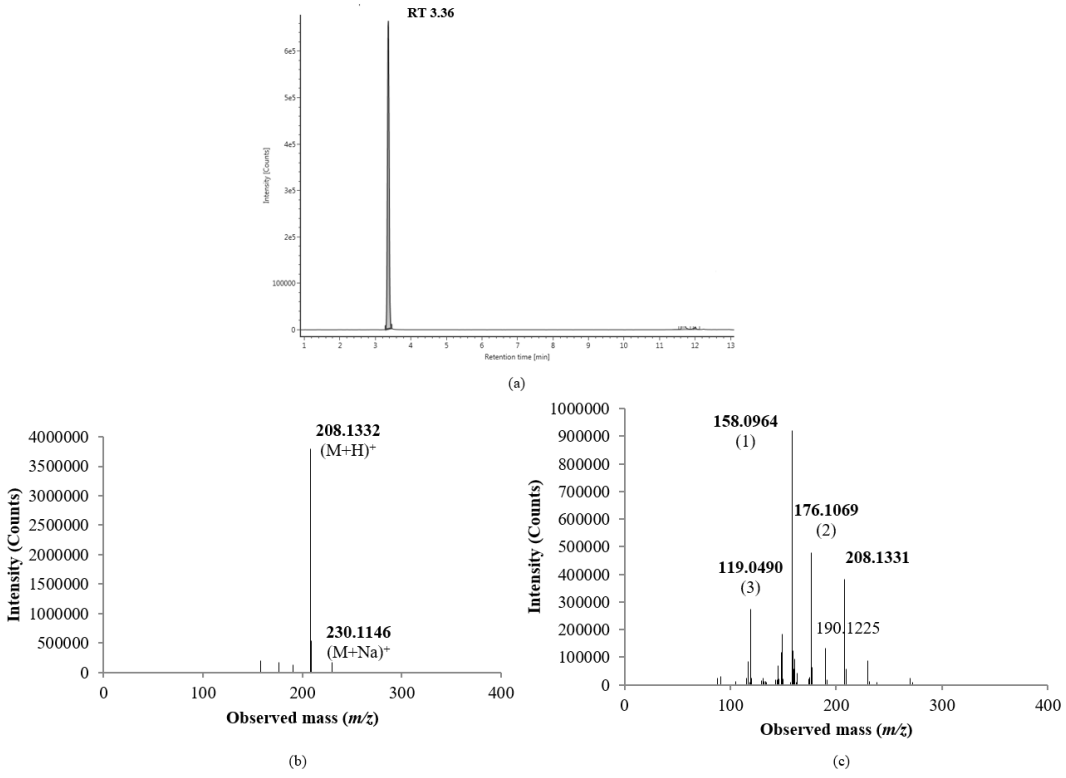


Figure 4.18 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 1 at (b) low energy (LE) and (c) high energy (HE).

This is possible due to the difference of 22 units between the two masses **(Figure 4.18b)**. Based on the obtained precursor ion, the value can be searched on HighResNPS by typing the mass with two decimals *"208.13"*, in this way four compounds are highlighted: MBDB, MDDMA, MDEA and mexedrone **(Figure 4.19)**. In terms of drug classes, the former ones belong to the phenethylamines category and the last one is a

representative of the cathinones group. Therefore, we can have a perception regarding the possible NPS classes that may be in this sample. However, the fragmentation is crucial to complete the analysis.

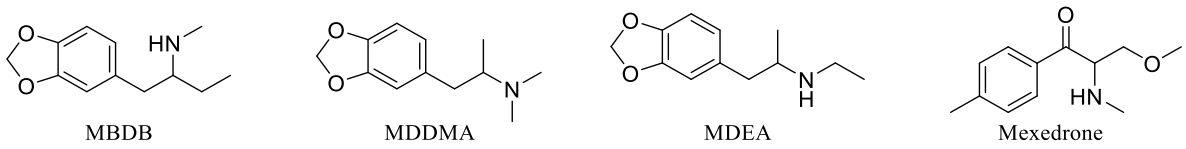


Figure 4.19 | Possible candidates on HighResNPS with a precursor ion at *m/z* 208.1332.

All the previous hints comprise fragment ions that can be compared with the ones observed experimentally **(Table 4.19)**. The high energy (HE) mass spectrum **(Figure 4.18c)** provides information regarding three collision-induced fragments, which are registered at *m/z* 158.0964, 176.1070 and 119.0491. In addition, if we look at the peak between the parent mass (*m/z* 208.1332) and fragment ion 2 (*m/z* 176.1070), we may observe the value 190.1225, which could suggest the existence of a carbonyl group owing to the loss of water (H_2O, 18 Da) [147]. Based on this assumption and taking into account the listed compounds, it would enable us to select merely one. In this case, mexedrone, since it is the only substance that contains this functional group in its chemical structure. Thus, it is necessary to assure that the obtained product ions comply with the ones available on the HighResNPS database. In this online library, the following fragments are reported: 119.0491, 158.0964 and176.1070 that completely agree with the ones in the spectrum. On the other hand, the remaining compounds correspond to ions that are not recorded (*e.g.* 105.0699 and 135.0440 **(Table 4.19 and Appendix O)**).

Table 4.19 The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
	F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
MBDB	$C_8H_7O_2$ *135.0441*	C_9H_{11} *119.0855*	$C_4H_{10}N$ *72.0808*	72.0808 119.0855 135.0441	-
MDDMA	$C_{10}H_{11}O_2$ *163.0754*	C_8H_9 105.0699	$C_8H_7O_2$ *135.0441*	177.0910 72.0808 105.0699	-
MDEA	C_8H_9 *105.0699*	$C_8H_7O_2$ 135.0441	$C_{10}H_{11}O_2$ *163.0754*	135.0441 163.0754	-
Mexedrone	**$C_{11}H_{12}N$** ***158.0964***	**$C_{11}H_{14}NO$** ***176.1070***	**C_8H_7O** ***119.0491***	**119.0491** **158.0964** **176.1070**	**119.0491** [148] **158.0964** [148] **176.1070** [148] 190.1226 [148]

Herein, it is important to interpret the occurrence of the aforementioned fragments: 119.0491 **(fragment a)** results in a benzoyl with a methyl substituent (loss of 89 Da), 158.0964 is related to a loss of 50 Da, which may indicate the loss of water with a consecutive disruption of the bond attached to the methoxy group and lastly, a cyclisation; and 176.1070 **(fragment b)** represents a loss of methanol (CH_3OH, 32 Da) **(Figure 4.20)**. Furthermore, the results were confirmed on mzCloud™; the product ions registered at 40 eV in positive mode and in the literature [148] were identical, proving that mexedrone is the compound present in this sample **(Table 4.19)**.

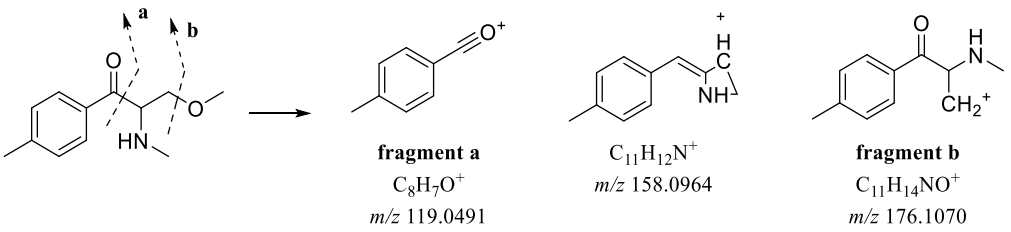


Figure 4.20 | Mexedrone: structure of its three fragments.

4.2.2 Sample 2

The **Figure 4.21a** shows a chromatographic peak at 5.48 min. In this case, it is observed that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 338.2224 ($C_{21}H_{27}N_3O$) with a mass error equal to -0.3 mDa and there is no evidence of sodium and potassium adducts **(Figure 4.21b)**. According to the obtained precursor ion, the value can be quested on HighResNPS by adding the mass with one decimal *"338.2"*, thus one compound is pointed up: ETH-LAD **(Figure 4.22)**, which fits into the indolalkylamines class. Of note, this substance was reported two times by different laboratories. In terms of fragmentation, the HE spectrum **(Figure 4.21c)** contributes with information at this level. Three collision-induced fragments were registered at *m/z* 237.1380, 208.0988 and 309.1837.

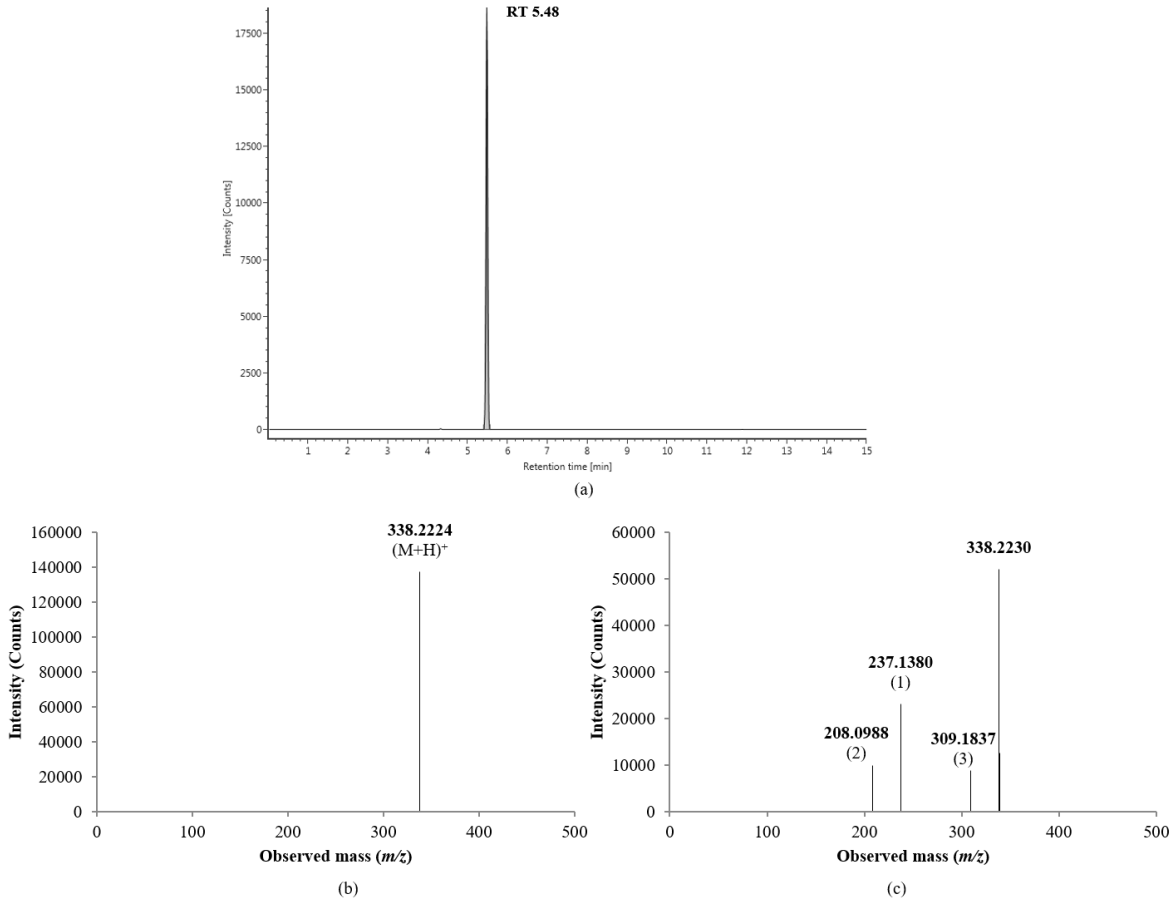


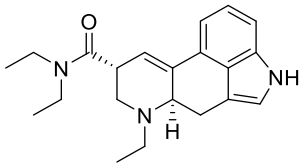
Figure 4.21 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 2 at (b) low energy (LE) and (c) high energy (HE).

On HighResNPS, the following product ions are found: 208.0995, 237.1386, 281.1648 and 309.1837 **(Table 4.20)**. In this way, the two laboratories consider the same first ions (208.0995 and 237.1386); however, the last two fragments do not match (281.1648 and 309.1837), accordingly.

Table 4.20 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for ETH-LAD [149].

Compound	HighResNPS database				mzCloud™ | CE= 40 eV	Literature
	Laboratory	F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
ETH-LAD	Denmark	**$C_{16}H_{17}N_2$** ***237.1386***	**$C_{19}H_{23}N_3O$** ***309.1835***	$C_{14}H_{12}N_2$ *208.0995*	-	208.0757 [149] **237.1386** [149] 281.1648 [149] **309.1836** [149]
	Australia	$C_{18}H_{21}N_2O$ 281.1648	**$C_{16}H_{17}N_2$** ***237.1386***	$C_{14}H_{12}N_2$ *208.0995*		

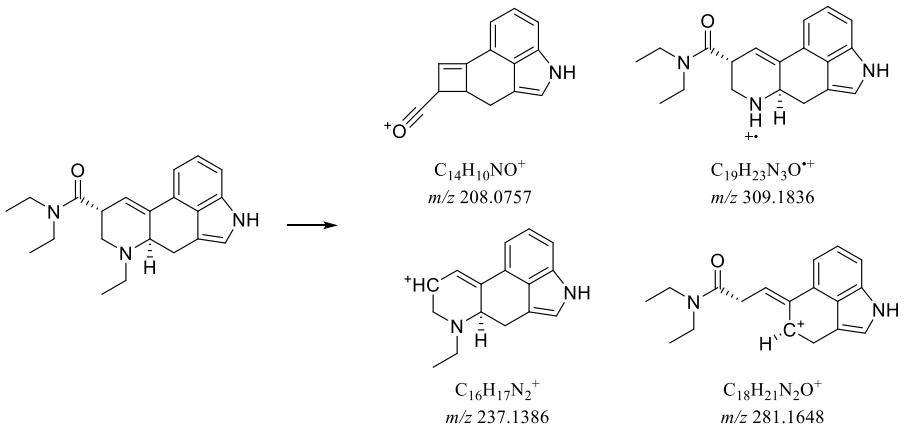
In addition, the HE spectrum takes into account 237.1386, 208.0995 and 309.1837. In general, we could affirm that the chances to be ETH-LAD are high, since the users are in accord with two product ions (*m/z* 208.0995 and *m/z* 237.1386) **(Table 4.20)**.



ETH-LAD

Figure 4.22 | Possible candidate on HighResNPS with a precursor ion at *m/z* 338.2227.

Regarding the fragmentation pathway of this molecule, it is complex, therefore it will not be considered in here. Nevertheless, the article of *Brandt et al.: Return of the lysergamides. Part III: Analytical characterization of N6-ethyl-6-norlysergic acid diethylamide (ETH-LAD) and 1-propionyl ETH-LAD (1P–ETH-LAD)* [149] gives an overview concerning this subject. Based on the previous article, it is possible to conclude with certainty that ETH-LAD is the compound present in this sample, since the product ions are identical to the ones published **(Table 4.20 and Figure 4.23)**.



$C_{14}H_{10}NO^+$
m/z 208.0757

$C_{19}H_{23}N_3O^{\bullet+}$
m/z 309.1836

$C_{16}H_{17}N_2^+$
m/z 237.1386

$C_{18}H_{21}N_2O^+$
m/z 281.1648

Figure 4.23 | ETH-LAD: structure of the fragments obtained from HighResNPS [149].

4.2.3 Sample 3

In the chromatogram, it is observed a peak corresponding to 5.15 min **(Figure 4.24a)**. The presence of a sodium adduct $(M+Na)^+$ at *m/z* 393.1956 indicates that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 371.2137 ($C_{22}H_{27}FN_2O_2$) with a mass error of 0.8 mDa. This is due to a difference of 22 units between the two masses **(Figure 4.24b)**.

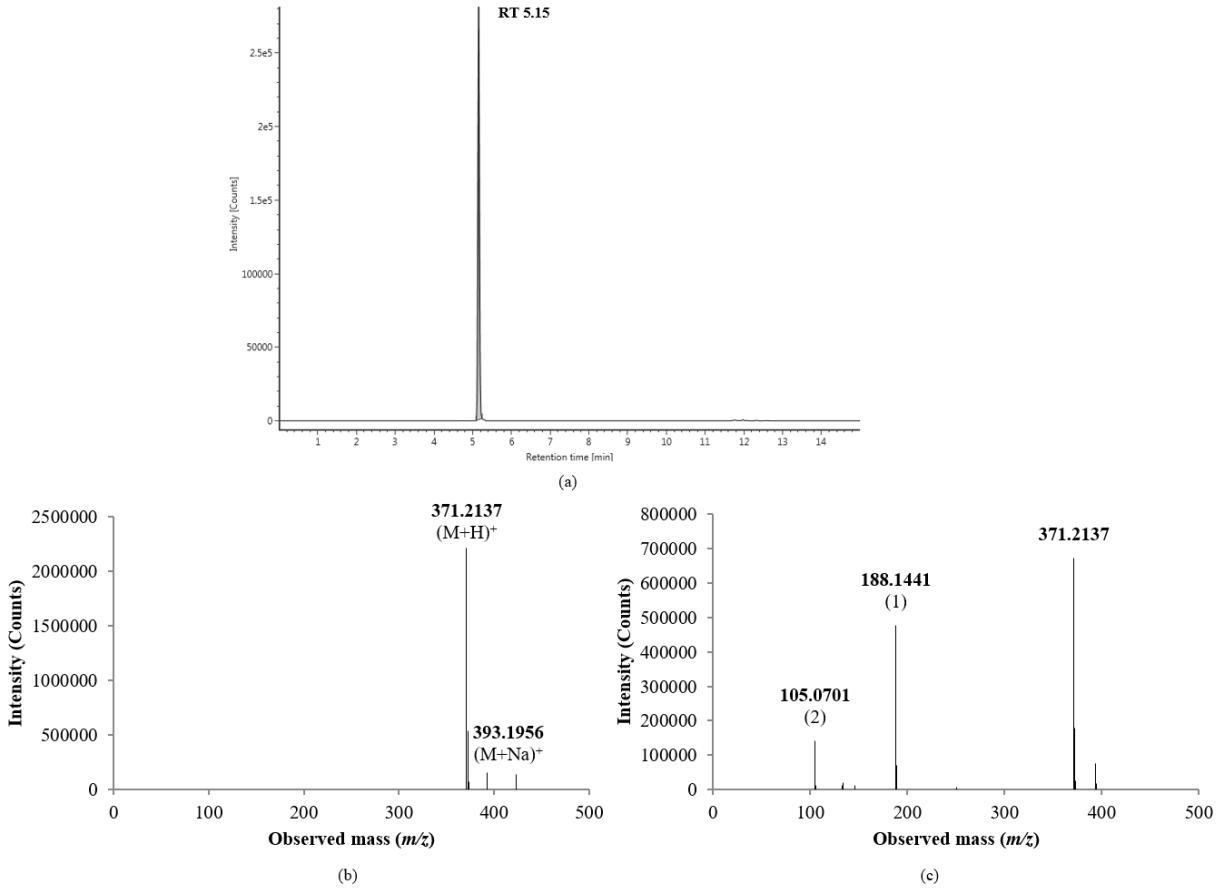


Figure 4.24 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 3 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, this value can be inspected on HighResNPS by typing the mass with two decimals *"371.21"*, in this sense three compounds are displayed: 2-fluoro methoxyacetylfentanyl (ocfentanil), 3-fluoro methoxyacetylfentanyl and 4-fluoro methoxyacetylfentanyl **(Figure 4.25)**, which are part of the opioids class, more specifically the fentanyls group.

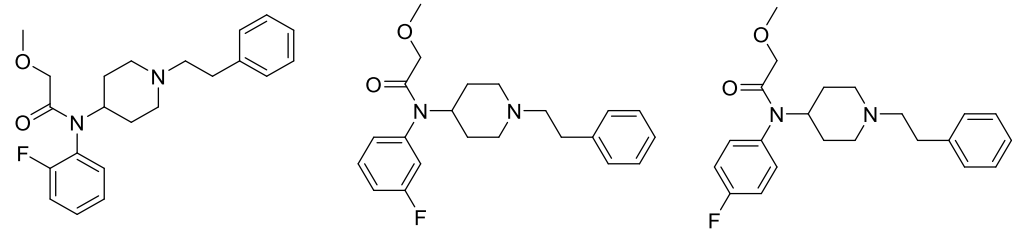


Figure 4.25 | Possible candidate on HighResNPS with a precursor ion at *m/z* 371.2129.

All the previous candidates contain fragment ions that can be compared with the ones observed experimentally **(Table 4.21)**. The HE spectrum **(Figure 4.24c)** provides information regarding two collision-induced fragments, which are registered at *m/z* 188.1441 and 105.0701. According to HighResNPS, the fragment ions associated to each hint are: 105.0699 and 188.1434 (for 2-fluoro methoxyacetylfentanyl), 105.0699, 188.1434 and 250.1238 (for 3-fluoro methoxyacetylfentanyl) **(Table 4.21)**. In terms of the characteristic product ions, 188.1434 **(fragment a)** results from the cleavage of the piperidine ring and the amide moiety, whereas 105.0699 **(fragment b)** derives from the previous ion and corresponds to a disruption between the alpha-carbon and the piperidine ring **(Figure 4.26)** [143].

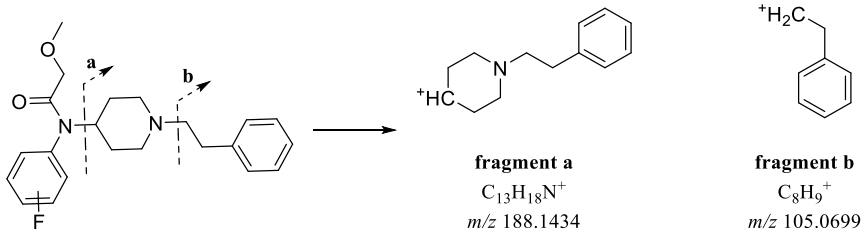


Figure 4.26 | The three hints: structure of the main fragments.

Regarding 4-fluoro methoxyacetylfentanyl no information on fragmentation is specified **(Table 4.21)**. In this way, it is possible to notice that both compounds encompass an identical chemical structure (*i.e.* positional isomers), thus they are more likely to produce equivalent fragments as verified previously. For this reason, it might be challenging to determine which one it is present in the sample. In this case, HighResNPS alone is not enough to contribute to a reliable result, therefore we need to resort to other approaches such as, purchase the three reference standards and/or perform NMR analysis, in order to localize the position (*-ortho*, *-meta* or *-para*) of the fluorine atom on the aniline ring.

Table 4.21 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
	F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
2-fluoro methoxyacetylfentanyl (Ocfentanil)	$C_{13}H_{18}N$ ***188.1434***	C_8H_9 ***105.0699***	-	***105.0699*** ***188.1434***	***105.0699*** [150] ***188.1434*** [150]
3-fluoro methoxyacetylfentanyl	$C_{13}H_{18}N$ ***188.1434***	C_8H_9 ***105.0699***	$C_{14}H_{17}FNO_2$ *250.1238*	***105.0699*** ***188.1434***	-
4-fluoro methoxyacetylfentanyl	-	-	-	-	-

4.2.4 Sample 4

In **Figure 4.27a**, a chromatographic peak is observed at 5.28 min. In this case, the protonated molecule $(M+H)^+$ corresponds to a *m/z* value 220.1700 ($C_{20}H_{25}N_3O$) with a mass error of 0.4 mDa and likewise the previous sample, there is no evidence of sodium and potassium adducts **(Figure 4.27)**. Based on the obtained precursor ion, this mass can be seek on HighResNPS by using the mass with one decimal *"220.1"*, in this way seven compounds are selected: 3-HO-PCE, 4-methyl-*N*,*N*-diethylcathinone, 4-methyl-*N*-ethyl-norpentedrone, NEiH, *N*-ethylhexedrone, NiPP and *N*-propylnorpentedrone **(Figure 4.28)**. In terms of drug classes, the former one belongs to the arylcyclohexylamines category and the last ones are representatives of the cathinones group. Therefore, we can be aware regarding the possible NPS classes that might be in this sample. In this sense, the fragmentation plays an important role. Not all the previous hints comprise fragment ions that can be compared

with the ones observed experimentally. Merely 4-methyl-*N*-ethyl-norpentedrone and *N*-ethylhexedrone contain fragments **(Table 4.22)**. The HE spectrum **(Figure 4.26c)** encompasses information regarding three collision-induced fragments, which are registered at *m/z* 202.1593, 146.0963 and 91.0541. In addition, if we look at the peak recorded at 202.1591 (low energy (LE) mass spectrum), it might indicate the presence of a carbonyl group owing to the loss of water (H_2O, 18 Da), which can be formed when a proton is attached to the oxygen atom [147]. Based on this premise and taking into account the listed compounds, it is possible to exclude 3-HO-PCE, since the remaining substances contain this functional group in their chemical structures.

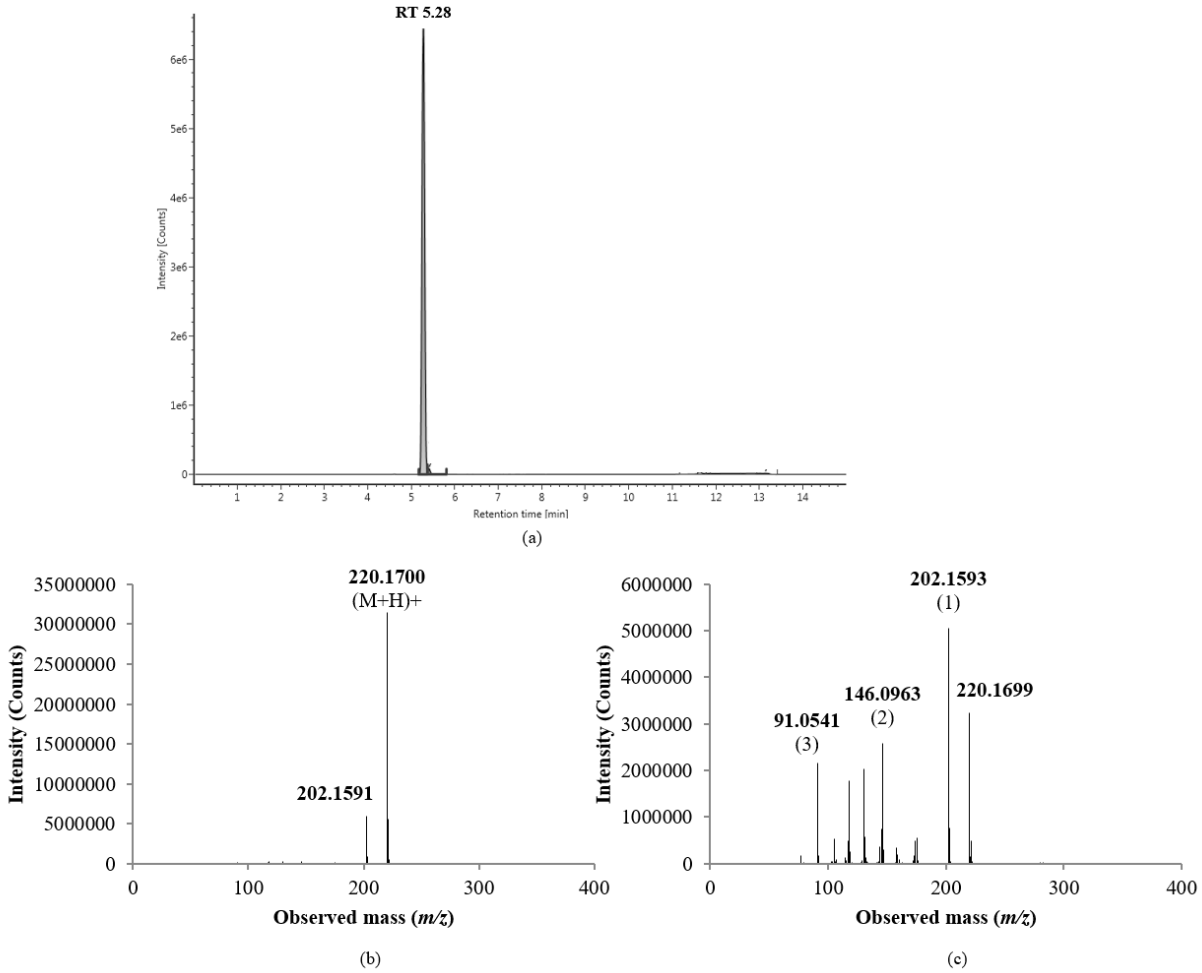


Figure 4.27 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 4 at (b) low energy (LE) and (c) high energy (HE).

Hence, it is necessary to guarantee that the obtained product ions comply with any of the compounds available on HighResNPS. It is important to notice that 4-methyl-*N*,*N*-diethylcathinone, NEiH, NiPP and *N*-propylnorpentedrone do not comprise fragments, in this way, a literature search might be essential to find information on fragmentation patterns that could facilitate the analysis process.

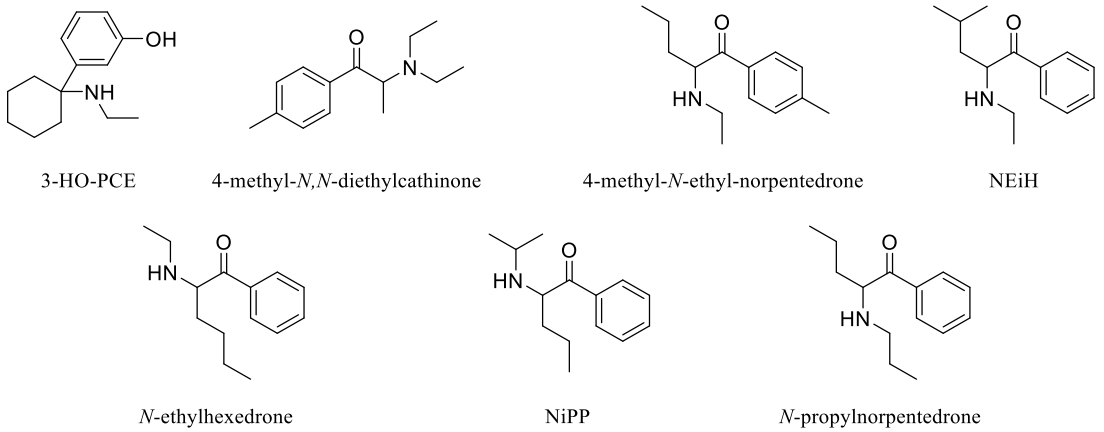


Figure 4.28 | Possible candidates on HighResNPS with a precursor ion at *m/z* 220.1696.

In the library, it is reported the following fragments: 105.0699, 144.0808 and 159.1042 (for 4-methyl-*N*-ethyl-norpentedrone) and 91.0542, 130.0651, 146.0964 and 202.1590 (for *N*-ethylhexedrone) **(Table 4.22)**. The latter compound contains more than one ion, since two laboratories included information on fragmentation.

Table 4.22 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
3-HO-PCE	Denmark	-	-	-	-	-
4-methyl-*N*,*N*-diethylcathinone	USA	C_9H_{11} *119.0855*	$C_6H_{14}N$ 100.1121	*$C_{10}H_{11}O$* *147.0804*	-	-
4-methyl-*N*-ethyl-norpentedrone	Finland	$C_{11}H_{13}N$ *159.1042*	$C_{10}H_{10}N$ *144.0808*	*C_8H_9* *105.0699*	-	105.0699 [151] 160.1121 [151] 175.1117 [151] **202.1590** [151]
NEiH	Denmark	-	-	-	-	-
N-ethylhexedrone	Denmark	C_7H_7 **91.0542**	$C_{10}H_{12}N$ **146.0964**	*$C_{14}H_{20}N$* ***202.1590***	***91.0542*** ***146.0964*** ***202.1590***	***91.0542*** [152] ***146.0964*** [152] ***202.1590*** [152]
	Australia	*$C_{14}H_{20}N$* ***202.1590***	$C_{10}H_{12}N$ **146.0964**	*C_9H_8N* *130.0651*		
NiPP	Denmark	-	-	-	-	-
N-propylnorpentedrone	Denmark	-	-	-	-	-

N/A: not applicable

According to the data, it might be assumed that *N*-ethylhexedrone is in the sample, which means that the fragments match accordingly **(Figure 4.29)**. However, this is not the case of 4-methyl-*N*,*N*-diethylcathinone and 4-methyl-*N*-ethyl-norpentedrone, none of their product ions correspond to the ones acquired. For NEiH, NiPP and N-propylnorpentedrone, the fragmentation was predicted based on scientific articles [151,153] **(Appendix P)**. Only one product ion (*m/z* 202.1590) is shared with *N*-ethylhexedrone and corresponds to the ones in the HE spectrum. Furthermore, the results were confirmed on mzCloud™; the product ions registered at 40 eV in positive mode and in the literature [152] were identical to *N*-ethylhexedrone **(Table 4.22)**, proving that it is the compound present in this sample.

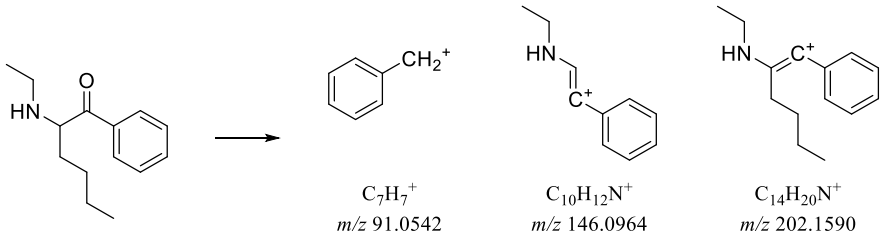


Figure 4.29 | *N*-ethylhexedrone: structure of its three fragments.

4.2.5 Sample 5

The **Figure 4.30a** illustrates a chromatographic peak at 6.54 min. The protonated molecule $(M+H)^+$ may correspond to a *m/z* value 375.2065 ($C_{24}H_{26}N_2O_2$) with a mass error of -0.2 mDa and no sodium and potassium adducts are observed **(Figure 4.30b)**. Based on the obtained precursor ion, the value can be searched on HighResNPS by typing the mass with three decimals *"375.206"*, in this way it is highlighted one compound: furanyl fentantyl **(Figure 4.31)**, which fits into the opioids class (*i.e.* fentanyls group).

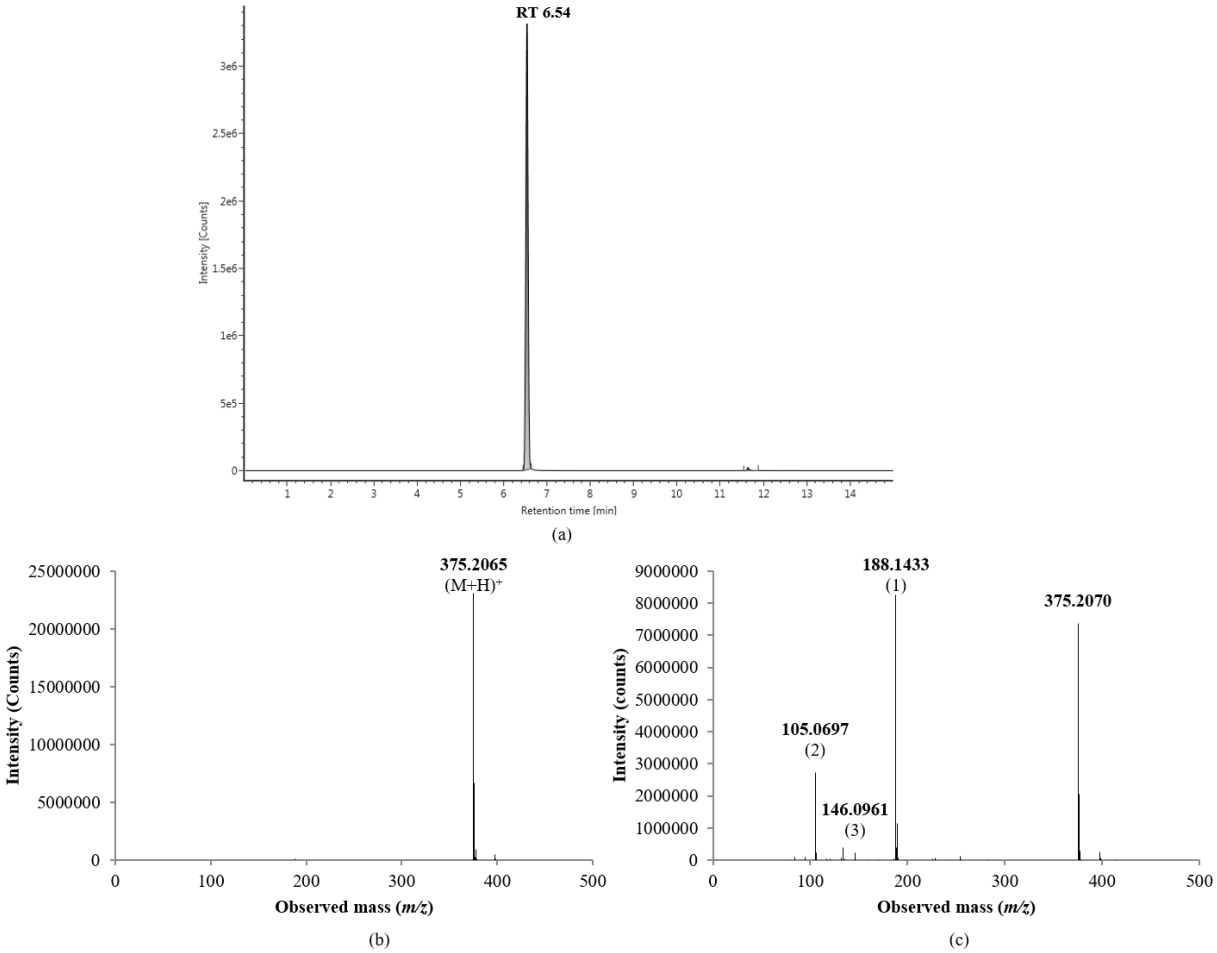
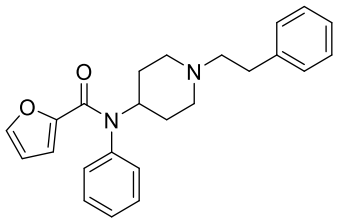


Figure 4.30 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 5 at (b) low energy (LE) and (c) high energy (HE).

Of note, this substance was reported four times by different laboratories **(Table 4.23)**. In terms of fragmentation, the HE spectrum **(Figure 4.30c)** contributes with information at this level. Three collision-induced fragments were registered at *m/z* 188.1433, 105.0697 and 146.0961.



Furanyl fentanyl

Figure 4.31 | Possible candidate on HighResNPS with a precursor ion at m/z 375.2067.

On HighResNPS, the following product ions are found: 105.0699, 146.0964 and 188.1434. In this way, the laboratories consider the same first ions (105.0699 and 188.1434); however, not all reported the last fragment (146.0964) **(Table 4.23)**.

Table 4.23 The product ions available on HighResNPS database, on mzCloud™ and in the literature for furanyl fentanyl [143].

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
Furanyl fentanyl	Australia	**$C_{13}H_{18}N$** ***188.1434***	**C_8H_9** ***105.0699***	**$C_{10}H_{12}N$** ***146.0964***	**105.0699** **146.0964** **188.1434**	**105.0699** [143] **188.1434** [143]
	Denmark	**$C_{13}H_{18}N$** ***188.1434***	-	-		
	Denmark	**$C_{13}H_{18}N$** ***188.1434***	-	-		
	Denmark	**$C_{13}H_{18}N$** ***188.1434***	**C_8H_9** ***105.0699***	-		

In terms of the fragment ions, 188.1434 **(fragment a)** results from the cleavage of the piperidine ring and the amide moiety, 105.0699 **(fragment b)** derives from the previous ion and corresponds to a disruption between the alpha-carbon and the piperidine ring and lastly, 146.0964 is due to degradation of the piperidine **(Figure 4.32)** [143].

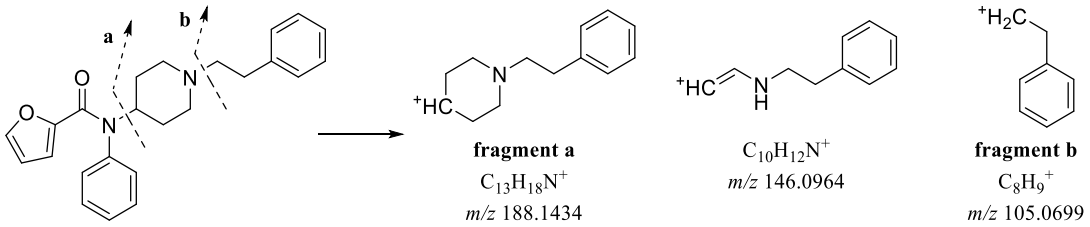


Figure 4.32 | Furanyl fentanyl: structure of the three fragments.

Furthermore, the result was confirmed on mzCloud™; the product ions registered at 40 eV in positive mode and in literature [143] were identical **(Table 4.23)**, proving that furanyl fentanyl is the compound present in this sample.

4.2.6 Sample 6

In **Figure 4.33a**, a predominant chromatographic peak is observed at 11.54 min. The presence of a sodium adduct $(M+Na)^+$ at *m/*z 399.2050 and a potassium adduct $(M+K)^+$ at *m/z* 415.1788 demonstrates that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 377.2232 ($C_{21}H_{29}FN_2O_3$) with a mass error of -0.3 mDa. This is possible due to the difference of 22 and 38 units between the parent mass and adducts. In addition, in-source fragments are also evident in the LE spectrum **(Figure 4.33b)**.

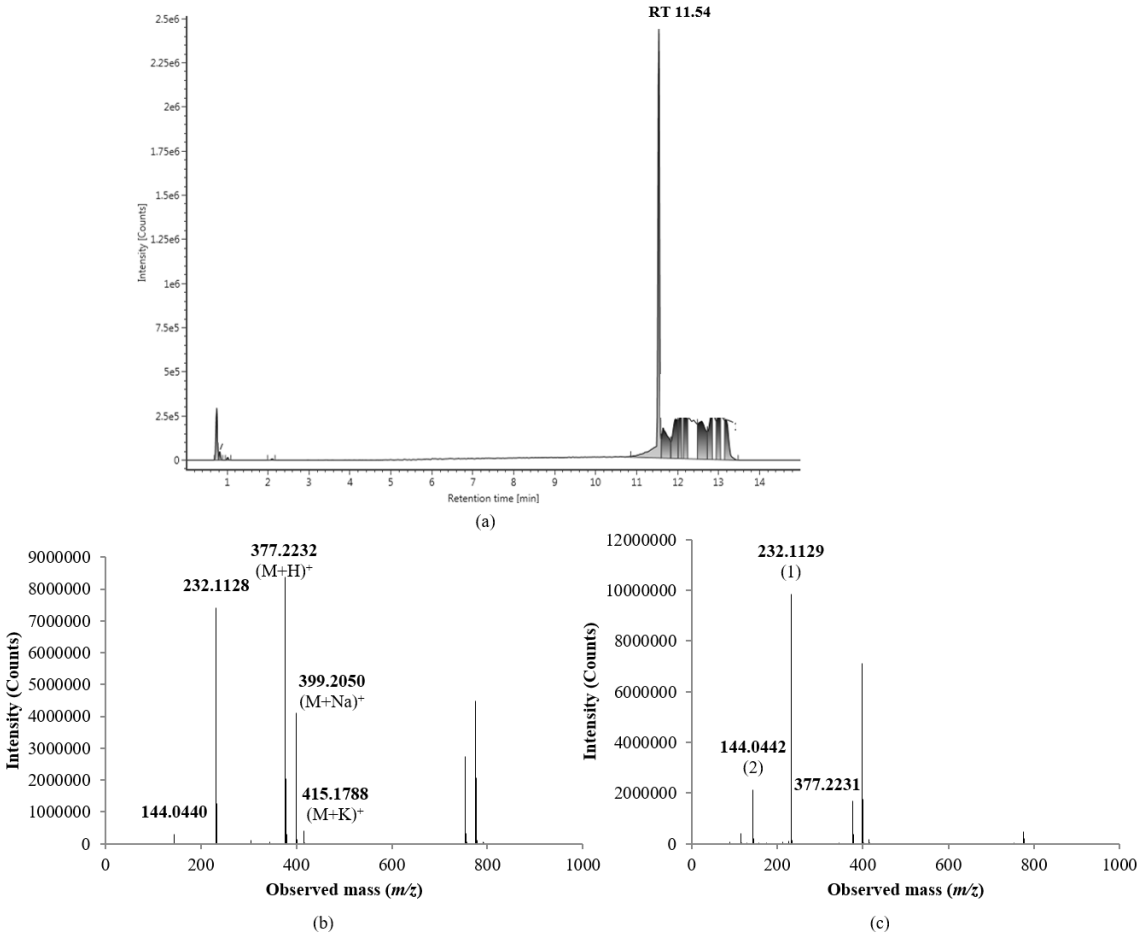
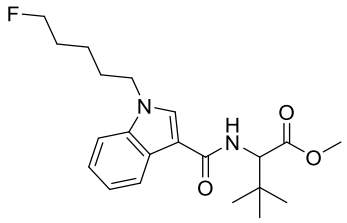


Figure 4.33 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 6 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, the value can be searched on HighResNPS by typing the mass with two decimals *"377.22"*, in this way one compound is pointed up: 5F-MDMB-PICA **(Figure 4.34)**, which fits into the cannabinoids class.



5F-MDMB-PICA

Figure 4.34 | Possible candidate on HighResNPS with a precursor ion at *m/z* 377.2235.

Of note, this substance was reported three times by different laboratories **(Table 4.24)**. In terms of fragmentation, the HE spectrum **(Figure 4.34c)** contributes with information at this level. Two collision-induced fragments were registered at *m/z*: 232.1129 and 144.0442. On HighResNPS, the following product ions are found: 144.0444 and 232.1132. In this way, the laboratories consider the same ions **(Table 4.24)**.

Table 4.24 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for 5F-MDMB-PICA [154].

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
5F-MDMB-PICA	Australia	**$C_{14}H_{15}FNO$** ***232.1132***	**C_9H_6NO** ***144.0444***	-		
	Denmark	**$C_{14}H_{15}FNO$** ***232.1132***	**C_9H_6NO** ***144.0444***	-	**144.0444** **232.1132**	**144.0444** [154] **232.1132** [154]
	Denmark	**$C_{14}H_{15}FNO$** ***232.1132***	**C_9H_6NO** ***144.0444***	-		

In terms of fragmentation, 232.1132 **(fragment a)** results from the core with a carbonyl group and the tail section, whereas 144.0444 **(fragment b)** derives from the previous ion and corresponds to a disruption of the core from the tail section **(Figure 4.35)** [44].

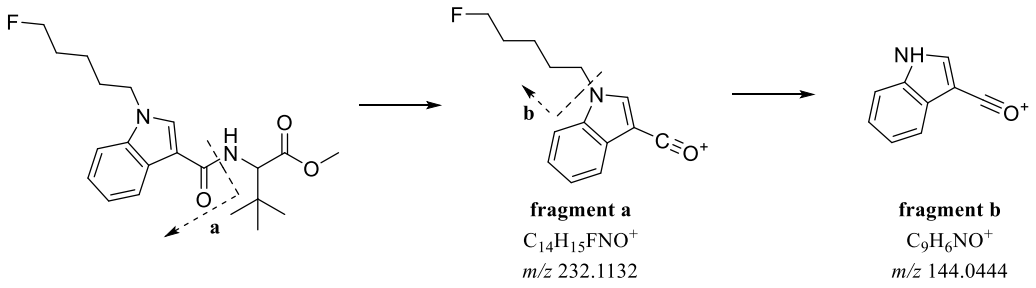


Figure 4.35 | 5F-MDMB-PICA: structure of the two fragments.

4.2.7 Sample 7

The **Figure 4.36a** illustrates a chromatogram. In this specific situation, an impurity is detected at 11.65 min, which is associated to the most intense peak. Hence, it was taken into account the second abundant at 8.73 min. The presence of a sodium adduct $(M+Na)^+$ at *m/z* 351.0438 and a potassium adduct $(M+K)^+$ at *m/z* 367.0178 demonstrates that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 329.0620 ($C_{16}H_{13}ClN_4S$) with a mass error of -0.2 mDa **(Figure 4.36b)**.

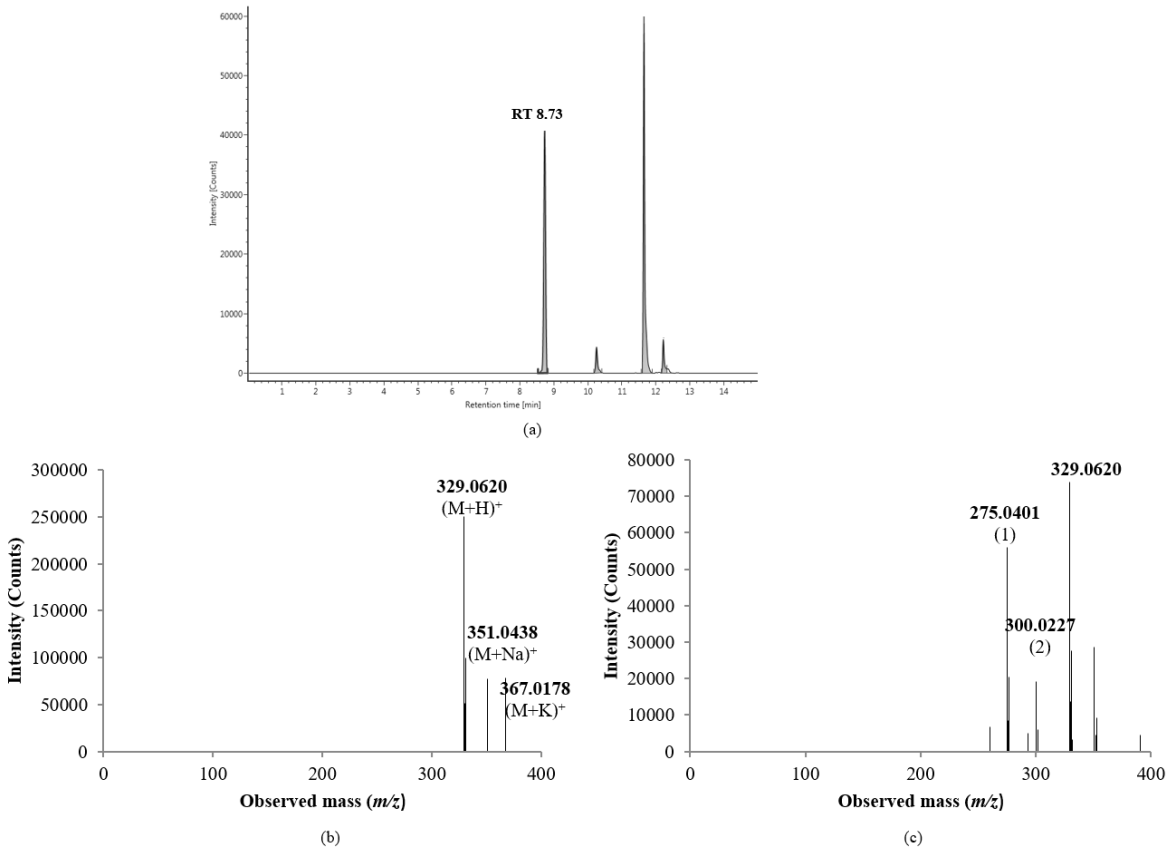
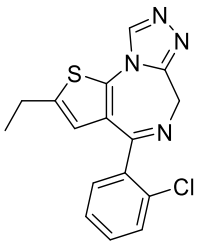


Figure 4.36 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 7 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, the value can be checked on HighResNPS by typing the mass with one decimal *"329.0"*, in this way one compound is displayed: metizolam **(Figure 4.37)**, which fits into the benzodiazepines class.



Metizolam

Figure 4.37 | Possible candidate on HighResNPS with a precursor ion at *m/z* 329.0622.

Of note, this substance was reported two times by different laboratories. The following product ions are identified: 275.0404 and 300.0231 **(Table 4.25)**. According to the HE spectrum **(Figure 4.36c),** two collision-induced fragments were registered at *m/z*: 275.0401 and 300.0227. It was observed that both match with the ones submitted in the library.

Table 4.25 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for metizolam.

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
Metizolam	Australia	**$C_{14}H_{12}ClN_2S$** ***275.0404***	**$C_{14}H_9ClN_4S$** ***300.0231***	-	-	-
	Denmark	**$C_{14}H_{12}ClN_2S$** ***275.0404***	**$C_{14}H_9ClN_4S$** ***300.0231***	-		

In terms of fragmentation, 275.0404 corresponds to a loss of 54 Da and 300.0231 is associated to a loss of -CH_2CH_3 (29 Da) [155] **(Figure 4.38)**.

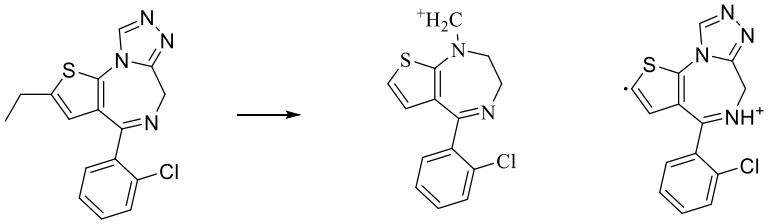


Figure 4.38 | Metizolam: structure of the two fragments.

Furthermore, mzCloud™ does not comprise this substance on its list. However, it is possible to conclude that metizolam might be present in this sample. In order to make sure, the reference standard may be purchased.

4.2.8 Sample 8

The **Figure 4.39a** shows a chromatographic peak at 11.68 min. The presence of a sodium adduct $(M+Na)^+$ at *m/z* 400.2005 and a potassium adduct $(M+K)^+$ at *m/z* 416.1743 demonstrates that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 378.2185 ($C_{20}H_{28}FN_3O_3$) with a mass error of -0.2 mDa. This is possible due to the difference of 22 and 38 units between the parent mass and respective sodium and potassium adducts **(Figure 4.39b)**.

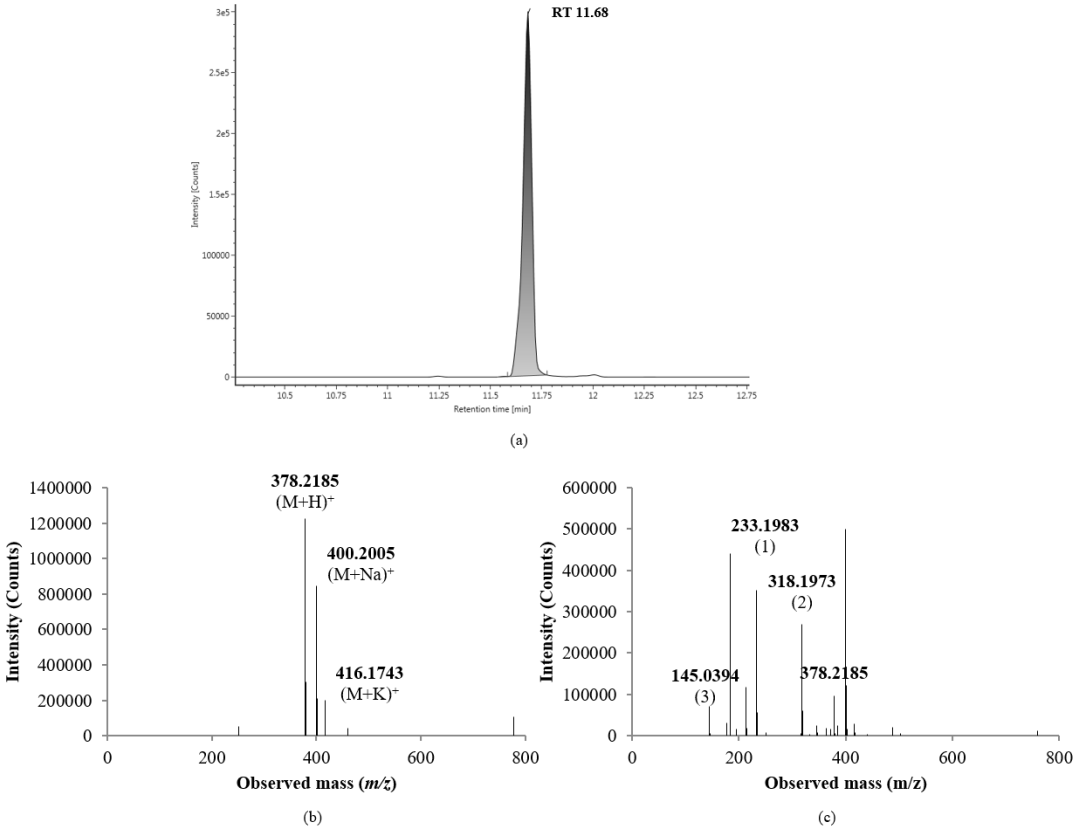


Figure 4.39 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 8 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, the value can be browsed on HighResNPS by typing the mass with three decimals *"378.218"*, therefore eight compounds are underlined: 2-fluoro ADB, 3-fluoro ADB, 4-fluoro ADB, 5-fluoro ADB, 5-fluoro AEB, 5-fluoro MDMB-PINACA, 5F-MDMB-P4AICA and 5F-MBMB-P7AICA **(Figure 4.40)**, which belong to the cannabinoids category. Some of the previous hints comprise fragment ions on HighResNPS database that can be compared with the ones observed experimentally.

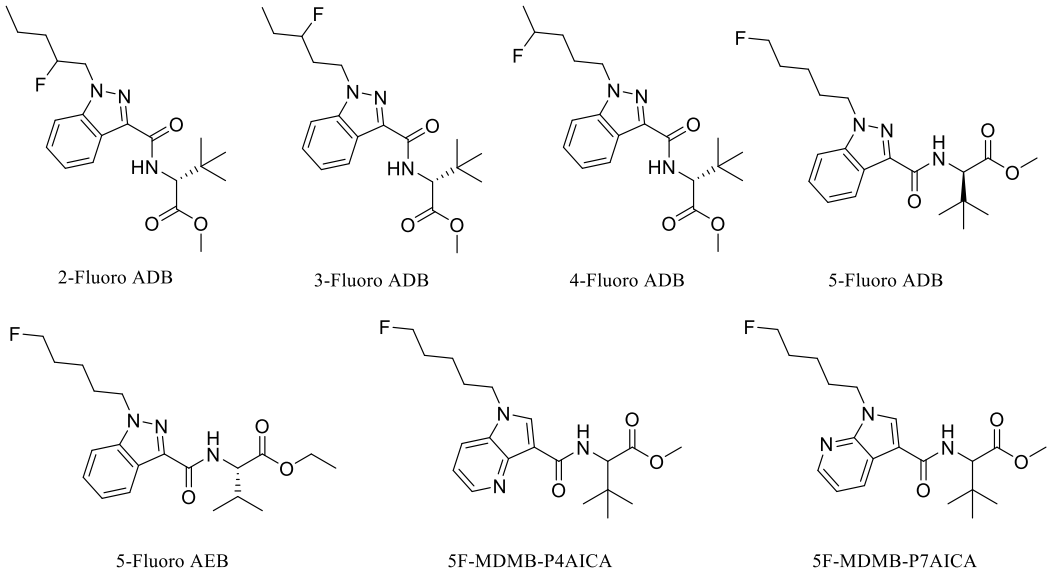


Figure 4.40 | Possible candidates on HighResNPS with a precursor ion at *m/z* 378.2187.

Regarding 5F-MDMB-P4AICA and 5F-MBMB-P7AICA no information on fragmentation is defined. In this way, it is possible to notice that four compounds encompass an identical chemical structure (*i.e.* 2-, 3-, 4- ,5-fluoro ADB, positional isomers), thus they are more likely to produce equivalent fragments. In addition, 5-fluoro AEB possesses a similar structure to 5-fluoro ADB, only differing in the moiety attached to the carboxyamide linker. When the product ions are not available, it is possible to predict them based on scientific articles [44] **(Appendix Q)**. The HE spectrum **(Figure 4.39c)** provides information regarding three collision-induced fragments, which are registered at *m/z* 233.1983, 318.1973 and 145.0394. The product ions were further compared with the ones available on HighResNPS: 145.0396, 233.1085 and 318.1976, in some situations it is also reported 69.0699, 213.1022 and 231.1618 **(Appendix R)**. This is observed since more than one laboratory submits the same compound and depending on the conditions, the fragments may suffer slight modifications **(Appendix R)**. In this sense, it is not possible to solve this issue using only HighResNPS, other procedures should be carried out in order to discover the compound present in this sample. Thus, mzCloud™ might assist in the interpretation, the reference standards of each substance might be purchased and/or NMR analysis may contribute to obtain a reliable result. Based on mzCloud™, it is possible to rule out one compound from the list (5-fluoro AEB), since it generates a product ion with a *m/z* 304.1820 **(Appendix R)**, which is not in agreement with the ones recorded in the HE spectrum. Nevertheless, it is important to notice that it contains two additional fragments (*m/z* 145.0396 and *m/z* 233.1085) that are identical to the other substances.

4.2.9 Sample 9

In the **Figure 4.41a**, a chromatographic peak is shown at 8.20 min. The presence of a sodium adduct $(M+H)^+$ at *m/z* 349.0626 proves that the protonated molecule $(M+H)^+$ may correspond to a *m/z* value 327.0811 ($C_{17}H_{12}ClFN_4$) with a mass error of 0.4 mDa.This is possible owing to the difference of 22 units between the masses **(Figure 4.41b)**.

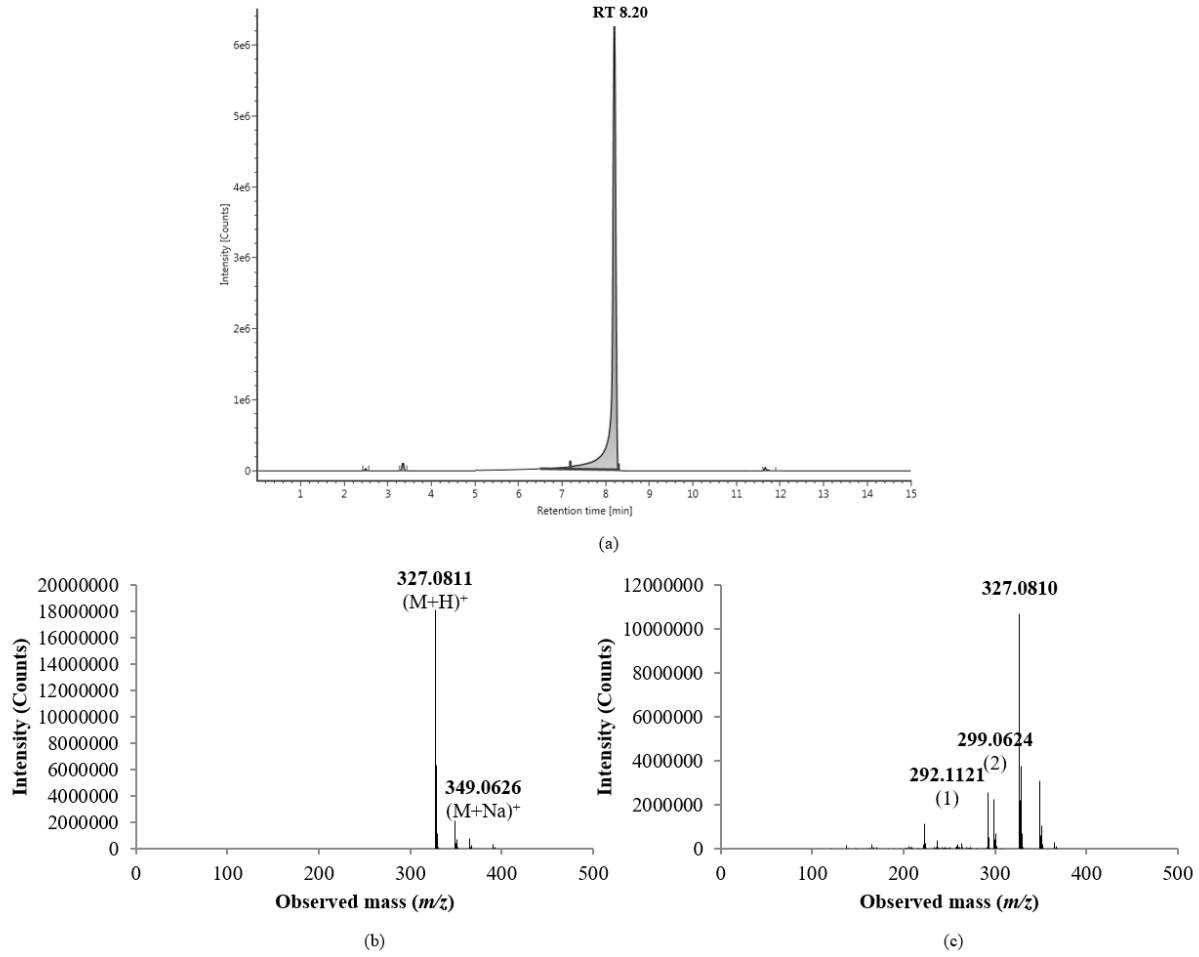
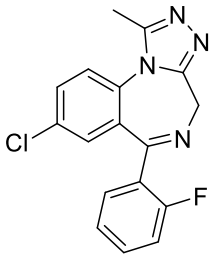


Figure 4.41 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 9 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, this value can be queried on HighResNPS by typing the mass with two decimals *"327.08"*, in this way only one compound is highlighted: flualprazolam, which makes part of the benzodiazepines class **(Figure 4.42)**.



Flualprazolam

Figure 4.42 | Possible candidate on HighResNPS with a precursor ion at *m/z* 327.0807.

This substance was submitted two times by different laboratories. In terms of fragmentation, the HE spectrum **(Figure 4.41c)** gives an overview of the two collision-induced fragments, which were registered at *m/z* 299.0624 and 292.1121. On HighResNPS, the following product ions are observed: 292.1119 and 299.0620, which are consistent with the ones obtained experimentally **(Table 4.26)**. Therefore, we could conclude that this benzodiazepine might be present in the sample.

Table 4.26 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for flualprazolam.

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
Flualprazolam	Denmark	**$C_{17}H_{13}FN_4$** ***292.1119***	-	-	-	-
	Denmark	**$C_{17}H_{13}FN_4$** ***292.1119***	**$C_{16}H_{11}ClFN_3$** ***299.0620***	-		

According to the fragments, it is possible to notice that 299.0620 is associated to a loss of CH_2N radical (28 Da), however its chemical structure is not clear (a suggestion is presented), whereas 292.1119 corresponds to a loss of Cl radical (35 Da) [155] **(Figure 4.43)**.

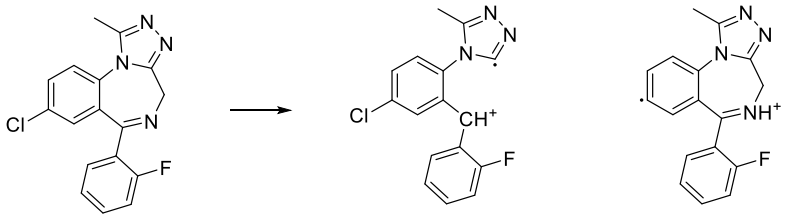


Figure 4.43 | Flualprazolam: structure of the two fragments.

4.2.10 Sample 10

The **Figure 4.44a** depicts an abundant chromatographic peak at 8.44 min. In this case, there is no evidence of sodium and potassium adducts and the protonated molecule $(M+H)^+$ corresponds to a *m/z* value 364.9690 ($C_{15}H_{10}BrClN_2O_2$) with a mass error equal to 0.2660 mDa **(Figure 4.44b)**. The obtained precursor ion can be further checked on HighResNPS by typing the mass with two decimals *"364.96"*, in this way two compounds are revealed: 3-hydroxyphenazepam and phenazepam 4-oxide **(Figure 4.45)**, both belonging to the benzodiazepines class. Regarding the latter substance no information on fragmentation is reported. However, it is possible to predict its fragmentation based on scientific articles [156] **(Appendix S)**.

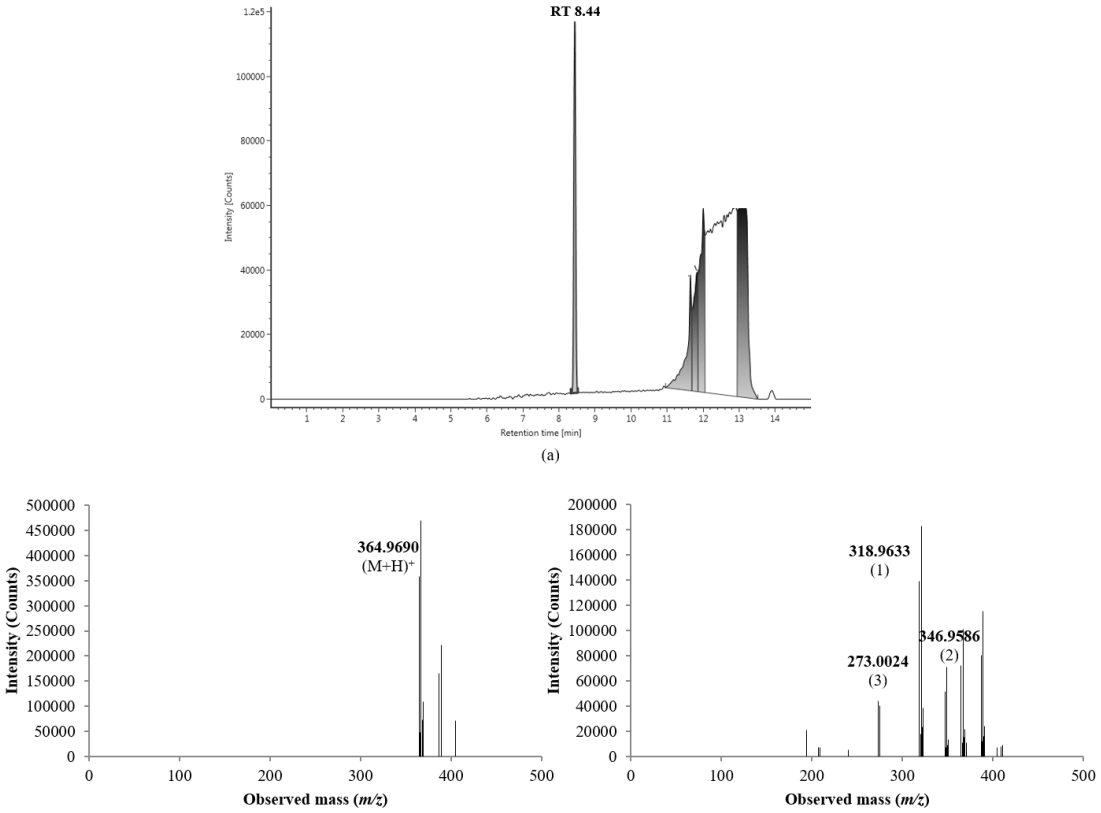
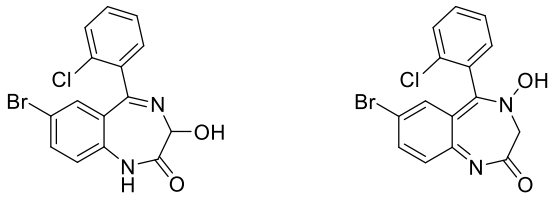


Figure 4.44 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 10 at (b) low energy (LE) and (c) high energy (HE).

Therefore, the three induced-collision fragments (318.9633, 346.9586 and 273.0024) in the HE spectrum **(Figure 4.44c)** will be compared with the data available for 3-hydroxyphenezepam by two different laboratories, which are identical: 273.0022, 318.9632 and 346.9581 **(Table 4.27)**. Hence, we might conclude that 3-hydroxy-phenazepam is the compound present in this sample **(Appendix S)**.



3-Hydroxyphenazepam Phenazepam 4-oxide

Figure 4.45 | Possible candidates on HighResNPS with a precursor ion at *m/z* 364.9687.

Nevertheless, in order to guarantee a proper answer, the two reference standards should be purchased.

Table 4.27 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	Laboratory	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
3-Hydroxy-phenazepam	Denmark	**$C_{14}H_9BrClN_2$** ***318.9632***	**$C_{15}H_9BrClN_2O$** **346.9581**	**$C_{13}H_{10}BrN_2$** **273.0022**	-	-
	Greece	**$C_{14}H_9BrClN_2$** ***318.9632***	**$C_{15}H_9BrClN_2O$** **346.9581**	**$C_{13}H_{10}BrN_2$** **273.0022**		
Phenazepam 4-oxide	Denmark	-	-	-	-	-

4.2.11 Samples 11 and 12

The chromatograms illustrate a peak at 4.17 min **(sample 11)** and 6.85 min **(sample 12) (Figure 4.46a)**. The protonated molecule $(M+H)^+$ corresponds to a *m/z* value 260.2010 ($C_{17}H_{25}NO$) with a mass error of 0.1 mDa **(sample 11)** and 260.2015 ($C_{17}H_{25}NO$) with a mass error equal to 0.6 mDa **(sample 12)**. For both samples, there is no indication of sodium and potassium adducts **(Figure 4.46b).**

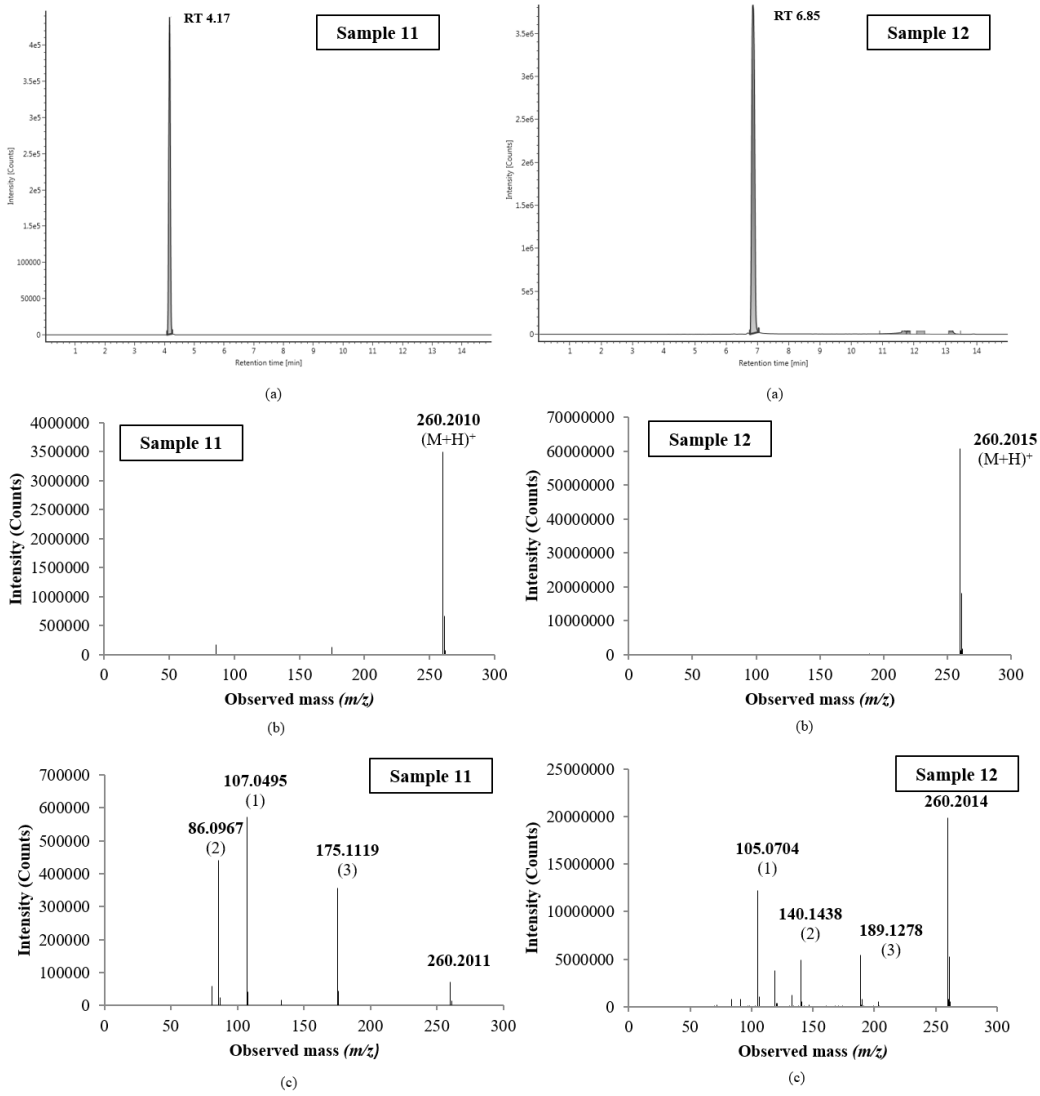
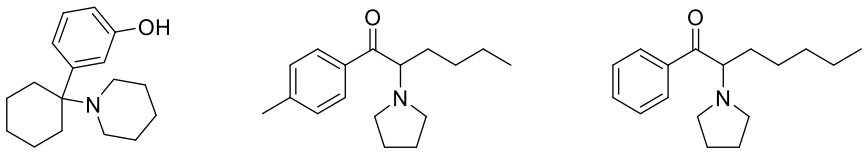


Figure 4.46 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown samples 11 and 12 at (b) low energy (LE) and (c) high energy (HE).

Based on the obtained precursor ion, the value can be searched on HighResNPS by typing the mass with one decimal *"260.2"*, therefore three compounds are highlighted: 3-HO-PCP, MPHP and PV8 **(Figure 4.47)**.



3-HO-PCP MPHP PV8

Figure 4.47 | Possible candidates on HighResNPS with a precursor ion at *m/z* 260.2009.

In terms of drug classes, the former one belongs to the arylcyclohexylamines category and the last ones are representatives of the cathinones group. Therefore, we can have an insight into the possible NPS classes that may be in this sample. However, the fragmentation is decisive to complete the analysis. All the previous hints comprise fragment ions that can be compared with the ones observed experimentally. For **sample 11**, the HE spectrum **(Figure 4.46c)** provides information regarding three collision-induced fragments, which are registered at *m/z* 107.0495, 175.1119 and 86.0967. In addition, if we look out there is no peak that could suggest the existence of a carbonyl group owing to the loss of water (H_2O, 18 Da) [147]. Based on this assumption and taking into account the listed compounds, it would enable us to select merely one: 3-HO-PCP. Thus, it is necessary to assure that the obtained product ions comply with the ones available on the HighResNPS database. On the library, it is reported the following fragments: 86.0964, 107.0491 and 175.1117, which correspond with the ones acquired **(Table 4.28)**. None of the fragments of the remaining two substances are compatible, a reason of exclusion **(Appendix T)**. Regarding the fragment ions, it is possible to notice that 107.0491 is formed by the dissociation of the aryl moiety with the cyclohexyl unit, 175.1117 corresponds to the bond break between the cyclohexyl unit and the piperidine ring and the last one, 86.0964 is the result of the previous cleavage, giving rise to a piperidium **(Figure 4.48)**.

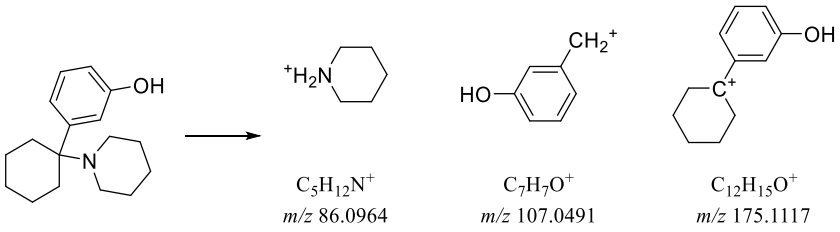


Figure 4.48 | 3-HO-PCP: structure of the three fragments.

For **sample 12**, the HE spectrum **(Figure 4.46c)** provides information regarding three collision-induced fragments, which are registered at *m/z* 105.0704, 140.1438 and 189.1278. In the library, the following fragments are reported for each substance: 107.0491, 86.0964 and 175.1117 (3-HO-PCP), 105.0699, 189.1274 and 140.1434 (MPHP) and 91.0542, 154.1590 and 189.1274 (PV8). According to the previous information, it is possible to notice that MPHP exhibits the exact same product ions, the fragments of PV8 only correspond to one peak (*m/z* 189.1278), while 3-HO-PCP does not report any. Therefore, 3-HO-PCP may be ruled out. Concerning the two remaining compounds, their fragments can be confirmed on mzCloud™ applying a collision energy 40 eV and in positive mode. Taking into account the fragmentation profile, it is observed that MPHP demonstrates akin results (*m/z* 105.0699 represents the base peak), whereas PV8 considers *m/z* 91.0542 the most intense peak and none of the aforementioned fragments are recorded. In this way, it might be inferred that MPHP is the unknown compound **(Appendix T)**.

Table 4.28 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	HighResNPS database			mzCloud™ | CE= 40 eV	Literature
	F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
3-HO-PCP	**C_7H_7O** ***107.0491***	**$C_5H_{12}N$** **86.0964**	**$C_{12}H_{15}O$** **175.1117**	-	-
MPHP	C_8H_9 *105.0699*	$C_{13}H_{17}O$ 189.1274	$C_9H_{18}N$ 140.1434	105.0699 140.1434 189.1274	105.0699 [157] 140.1434 [157] 189.1274 [157]
PV8	C_7H_7 *91.0542*	$C_{10}H_{20}N$ 154.1590	$C_{13}H_{17}O$ 189.1274	91.0542 154.1590 189.1274	91.0591 [157] 154.1590 [157] 189.1274 [157]

4.2.12 Sample 13

The **Figure 4.49a** represents an abundant chromatographic peak at 3.30 min. The presence of an in-source fragment (*m/z* 109.0452) demonstrates that the protonated molecule $(M+H)^+$ corresponds to a *m/z* value 182.1345 ($C_{11}H_{16}FN$) with a mass error of 0.6 mDa and there is no evidence of sodium and potassium adducts **(Figure 4.49b)**.

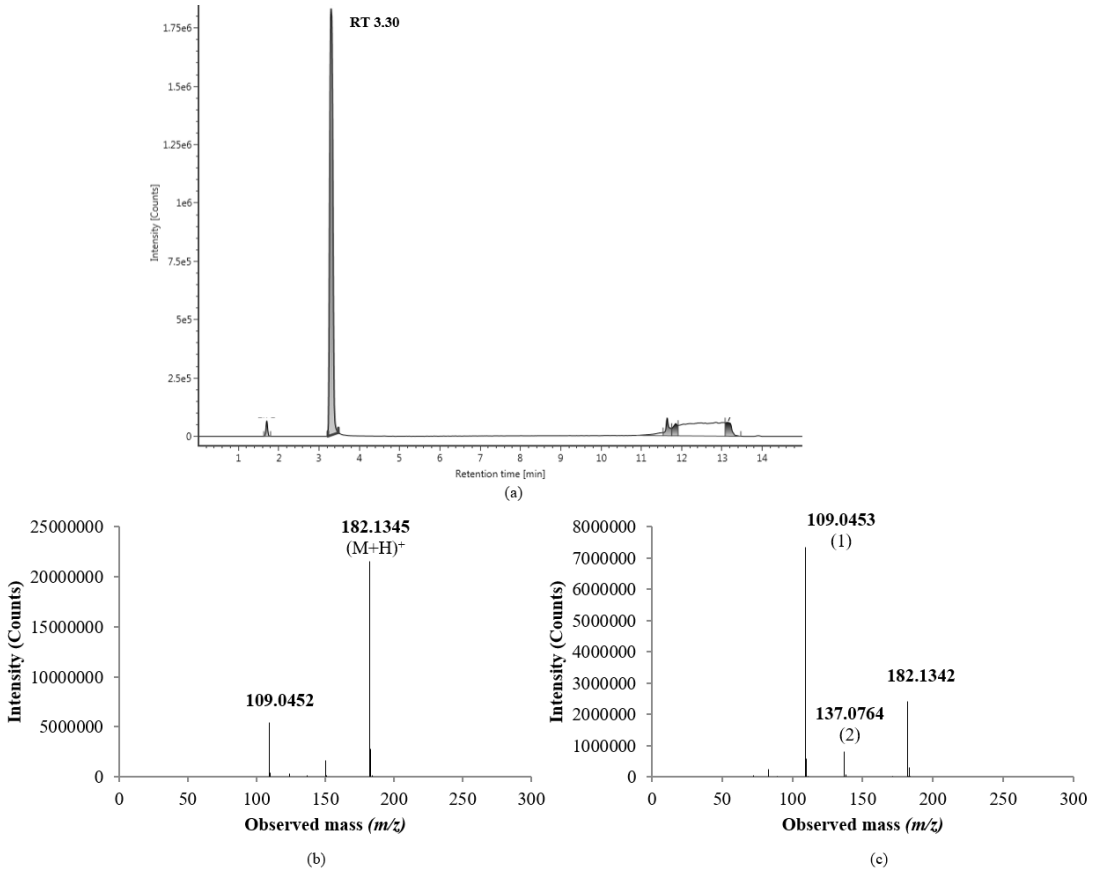


Figure 4.49 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 13 at (b) low energy (LE) and (c) high energy (HE).

The precursor ion can be investigated on HighResNPS by typing the mass with two decimals *"182.13"*, in this way two compounds are pointed up: 2-fluoroethamphetamine (2-FEA) and 3-fluoroethamphetamine (3-FEA) **(Figure 4.50)**, both phenethylamines. Of note, only 3-FEA comprises information on fragments, which are registered at *m/z* 109.0448 and 137.0761 **(Table 4.29)**. Comparing these values with the ones obtained in the HE spectrum **(Figure 4.49c)**, it is possible to notice the existence of two induced-collision fragments at *m/z* 109.0453 and 137.0764, which are in agreement with the ones recorded.

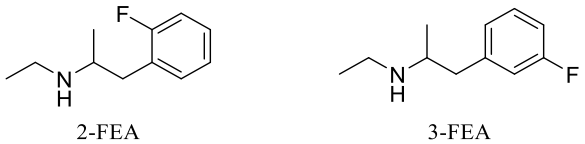


Figure 4.50 | Possible candidates on HighResNPS with a precursor ion at *m/z* 182.1339.

However, we are not able to provide a proper result, since both substances are positional isomers, which makes their distinction challenging **(Figure 4.51)** [110].

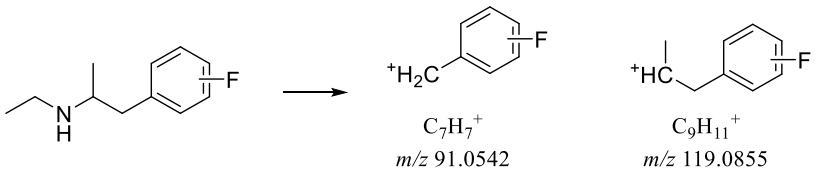


Figure 4.51 | The two hints: structure of the main fragments based on scientific articles [110].

In this way, it might be necessary to resort to other methods such as, purchase the two reference standards and/or carry out NMR experiments, in order to define the position of the fluorine atom on the aromatic ring.

Table 4.29 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	HighResNPS database			mzCloud™ | CE= 40 eV	
	F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	Literature
2-FEA	-	-	-	-	-
3-FEA	C_7H_6F *109.0448*	$C_9H_{10}F$ 137.0761	-	-	-

4.2.13 Sample 14

In **Figure 4.52a**, it is illustrated a chromatographic peak at 3.07 min. The presence of in-source fragments at *m/z* 180.0578 and 145.0885 confirms that the protonated molecule $(M+H)^+$ corresponds to a *m/z* value 198.0685 ($C_{10}H_{12}ClNO$) with a mass error of 0.45 mDa **(Figure 4.52b)**.

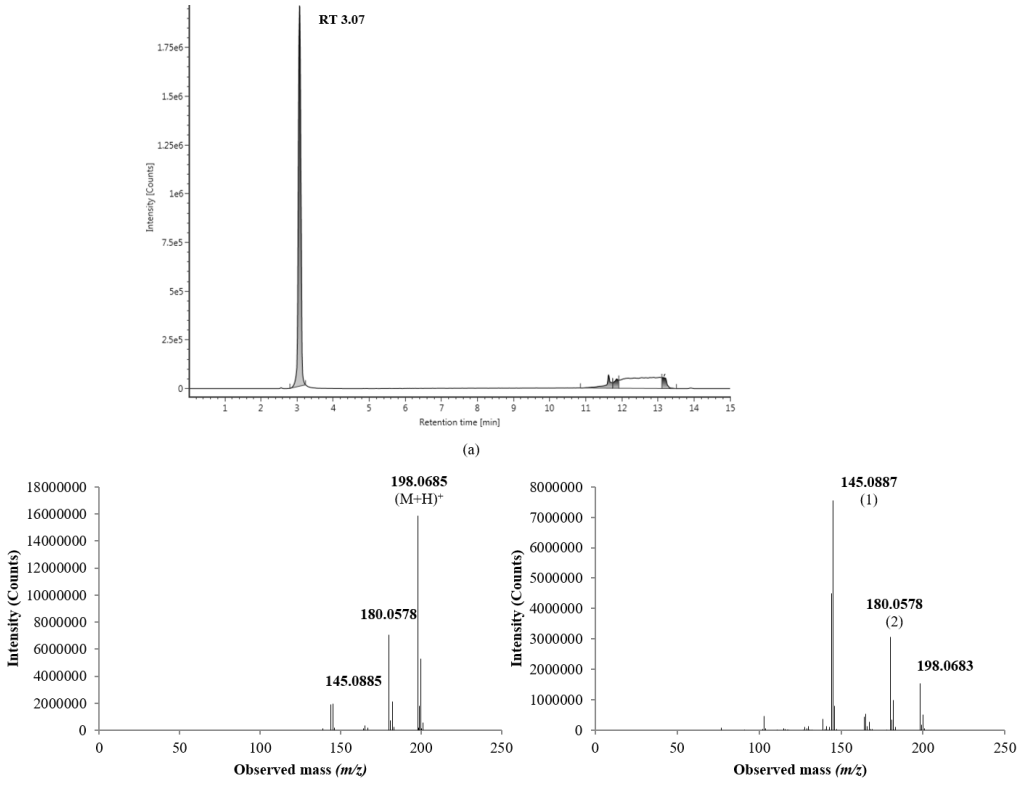
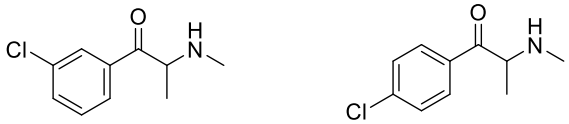


Figure 4.52 | (a) LC-QTOF-MS total ion chromatogram (TIC), MS^E collision spectra of the unknown sample 14 at (b) low energy (LE) and (c) high energy (HE).

The precursor ion can be further browsed on HighResNPS by typing the mass with one decimal *"198.0"*, therefore two compounds are found: 3-CMC and 4-CMC **(Figure 4.53)**, both belonging to the cathinones class.



3-CMC 4-CMC

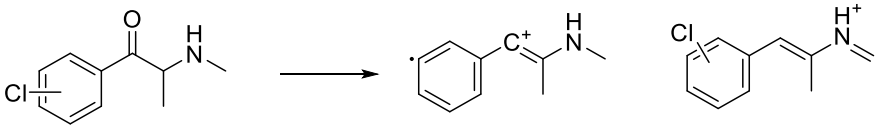
Figure 4.53 | Possible candidates on HighResNPS with a precursor ion at *m/z* 198.0680.

Of note, only 4-CMC comprises information on fragments, which are registered at *m/z* 145.0886, 180.0574, 144.0808 and 139.0309. It is important to mention that this substance was reported by two laboratories that agree in the first two values, however the latter ones suffer some variations **(Table 4.30)**.

Table 4.30 | The product ions available on HighResNPS database, on mzCloud™ and in the literature for each candidate.

Compound	Labora-tory	HighResNPS database			mzCloud™ | CE= 40 eV	Liter-ature
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments	
3-CMC	Denmark	$C_{10}H_{10}N$ 144.0808	-	-	-	-
4-CMC	Australia	**$C_{10}H_{11}N$** ***145.0886***	**$C_{10}H_{11}ClN$** **180.0574**	$C_{10}H_{10}N$ 144.0808	-	-
	Greece	**$C_{10}H_{11}N$** ***145.0886***	**$C_{10}H_{11}ClN$** **180.0574**	C_8H_8Cl 139.0309	-	

In addition, it is also observed a loss of water (18 Da), which results in a *m/z* 180.0578; this may confirm the existence of a carbonyl functional group [147]. In comparison with the two induced-collision fragments at *m/z* 145.0887 and 180.0578 **(Figure 4.52c)**, it is possible to conclude that these two are consistent with the ones recorded. Nevertheless, 3-CMC and 4-CMC contain an identical chemical structure, thus it is difficult to suggest which one is present in the sample due to their alike fragmentation **(Figure 4.53)**.



$C_{10}H_{11}N^{\bullet+}$ $C_{10}H_{11}ClN^{+}$
m/z 145.0886 *m/z* 180.0575

Figure 4.54 | The two hints: structure of the main fragments based on scientific articles [144].

In this case, it would be recommended to find other alternatives that might help to identify the right substance such as, perform NMR experiments and/or buy the two reference standards to determine the position of the chlorine atom on the aromatic ring.

5 CONCLUSION AND FUTURE PERSPECTIVES

The purpose of this dissertation was to improve and test a universal online library named HighResNPS for new psychoactive substances when reference standards are not readily available using UHPLC-QTOF-MS. The first part of this thesis consisted of ensuring that the database contained accurate information on the following parameters: drug class, IUPAC name and exact masses, including both precursor and fragment ions. In this way, the creation of two features allowed to successfully assigning 290 mass errors, to find some of the absent molecular formulae and to revise 61 systematic names. In addition, the InChIkey, which can be generated through the IUPAC name, is referred to as an important criterion for pinpoint compounds, since it is unique to a specific substance. The absence of this parameter may lead to a challenge, especially when dealing with isomers due to their similar fragmentation patterns, which makes their identification laborious. Furthermore, 333 entries reported by EMCDDA and RESPONSE libraries were excluded from the database, since they were not adding any valuable information. The previous entries comprised only exact mass, molecular formula, systematic name, InChIkey and drug class, which is termed as non-experimental data. However, the process was applied exclusively to compounds presenting more than one entry.

In forensic analysis, fragmentation plays an important role in data analysis providing the scientist with structural information of a certain compound that might be helpful especially when reference standards are not available. In this sense, a combination of accurate-mass and fragmentation patterns allow the identification of substances present in the samples. Taking into account the NPS categories, it is essential to stress that not all the groups of compounds are easy to predict fragmentation owing to their structure complexity. The steady emergence of fentanyl analogues and synthetic cannabinoids on the drug market, and recently the occurrence of several acute intoxications and fatal cases lead us to predict the fragmentation of compounds within these two NPS classes. Therefore, two sub-libraries were created containing 51 and 215 compounds, respectively. The common fragmentation patterns were described for fentanyl analogues. It was verified that these compounds cleave between the amide moiety bond and the piperidine ring. Further cleavages on the piperidine ring and in the C-N amide bond may also take place. This study came to prove that the first fragments coincide frequently with the ones reported among laboratories and, to a lesser extent, second and third product ions. Thus, the fragmentation pathways can be employed on future analogues, since it contributes to a general knowledge of the type of fentanyls we have at hand. In case of synthetic cannabinoids, this class of compounds is represented by a core, a tail section, a linker and a linked group, the variances among them may lead to different sub-groups. It was confirmed that the cleavage of the tested molecules mainly took place on the bond linking the carbon atom of the carbonyl group and the ring or the NH group attached to the ring. Furthermore, the predicted fragmentation might be useful in determining and interpreting future analogues. Based on the seven examples given for each sub-group, we concluded that the first product ion is imperative to distinguish the type of SCs and the remaining two fragment ions contribute with supplemental information on the chemical composition. It is worth noting that this approach is not always perfect due to the chemical modifications that may occur. For example, JWH-175 contains a methylene group as a linker, which does not comply with the fragmentation principles established.

Regarding the database progress, we have noticed an evolution on the number of compounds and fragmentation. In March 2019, it comprised 1251 compounds, meaning that 156 substances were included since the beginning of the validation process. Besides that, it is composed by 775 NPS containing product ions, whereas 476 were still absent. In addition, 273 compounds with fragments were recorded by more than one laboratory and 99.6% shared at least one fragment. Throughout the database, it was possible to witness the presence of 349 isomer groups. Of note, the positional isomers may pose a problem, since they differ merely in the position of a substituent and the fragmentation pattern is identical. On the other hand, some compounds may encompass

the exact same mass; nevertheless, it is possible to differentiate them thanks to a clearly difference in their fragment values, therefore promoting their identification.

HighResNPS can be converted into an in-house library. In this thesis, a different approach is described to interpret the analytical information. It is important to highlight that it might be useful to import the database when dealing with complex samples (*i.e.* blood matrices); however, in the case of seizures, it is recommended to use it as an online mass spectral database, since it allows an easy access to the last updates, without the need to download a new version every time a novel entry/compound comes in.

The second part of this thesis focused on the analysis of 14 unknown substances. The proposed workflow consisted of searching the precursor ion on the database using a mass with at least one decimal, which allowed to reduce the false positives. In addition, the fragmentation profile helped in ruling out some of the listed compounds. Thus, a possible chemical structure might be elucidated. Nonetheless, the latter step is not always attainable, especially when positional isomers come into play. In this sense, it might be necessary to resort to other methodologies such as, perform NMR analysis and/or purchase reference standards (*i.e.* comparing the Rt of all compounds), in order to determine the position of a certain substituent. Furthermore, a tentative identification and confirmation of the tested samples might be possible thanks to the use of mzCloud™ (*i.e.* spectra comparison) and search of scientific articles. Hence, HighResNPS may provide a preliminary identification concerning the NPS class of the analysed sample and increase the chances of finding the right compound. Finally, the success of the database relies on how active the users are. As a universal library, it is fundamental that the involved laboratories share their results, so that the analysis can be more accurate.

In terms of future perspectives, it would be interesting to predict the retention time (Rt) for the current and future compounds and on a more advanced level, use software tools to envisage the fragmentation for each drug class available on the database.

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7 APPENDICES

Appendix A | 58 novel compounds monitored in six drug websites. The green colour represents where the substances were found.

Compound	erowid.org	Chem.eu	NMS labs	forendex	EWS Forum	drugs-forum.com	Drug class	Monitoring date
Bromadol							ACH	25/10/2018
MIPLA							I	25/10/2018
4-ANPP							P&P	26/10/2018
m-Methyl methoxyacetyl fentanyl							OPD	26/10/2018
p-Methyl methoxyacetyl fentanyl							OPD	26/10/2018
trans-3-Methyfentanyl							OPD	26/10/2018
Delorazepam							BZD	26/10/2018
1,3-Benzodioxolylbutanamine (BDB)							PEA	26/10/2018
4-Ethylamphetamine							PEA	26/10/2018
4-Fluoro MDMB-PICA metabolite 7							CB	26/10/2018
ADB-PINACA pentanoic acid metabolite							CB	26/10/2018
MAB-CHMINACA metabolite M2							CB	26/10/2018
MAB-CHMINACA metabolite M3							CB	26/10/2018
MDMB-FUBINACA metabolite M1							CB	26/10/2018
AM2201 8-quinolinyl carboxyamide							CB	29/10/2018
4-HO-MPT							I	29/10/2018
N-Methylephedrine							PEA	29/10/2018
2-Methylacetylfentanyl							OPD	29/10/2018
4-HO-DMT							I	29/10/2018
AMB-4en-PICA							CB	29/10/2018
4-Fluoro-cyclopropylbenzylfentanyl							OPD	29/10/2018
2-Fluoroethamphetamine; 2-FEA							PEA	29/10/2018
3,4-Methylenedioxy-U-47700							OPD	29/10/2018
3F-α-PVP							CAT	29/10/2018
25E-NBOH							PEA	29/10/2018
4-Desoxymescaline							PEA	01/11/2018
Tapentadol							OPD	01/11/2018
1,4-Butanediol							UNK	01/11/2018
4-MeO-DMT							I	01/11/2018
N,*N*-Dibutyltryptamine							I	01/11/2018
Pyr-T (*N*,*N*-tetramethylenetryptamine)							I	01/11/2018
Methylbutyltryptamine (MBT)							I	01/11/2018
Ethylisopropyltryptamine (EiPT)							I	01/11/2018
2CBFly-NBOMe (NBOMe-2C-B-FLY)							PEA	01/11/2018

ACH: Arylcyclohexylamines, I: Indolalkylamines, P&P: Piperidines & pyrrolidines, OPD: Opioids, BZD: Benzodiazepines, PEA: Phenethylamines.

Appendix A | 58 novel compounds monitored in six drug websites. The green colour represents where the substances were found (continued).

Compound	erowid.org	Chem.eu	NMS labs	forendex	EWS Forum	drugs-fo-rum.com	Drug class	Monitoring date
N-Ethyltrypta-mine (NET)							I	01/11/2018
CB-52							CB	01/11/2018
CB-25							CB	01/11/2018
BAY 38-7271							CB	01/11/2018
Harmaline							P&E	01/11/2018
AKB-N1							CB	01/11/2018
6-Methyl-MDA							PEA	01/11/2018
N-Methyltryptamine							I	01/11/2018
2,5-Dimethoxy-4-ethylamphetamine (DOET)							PEA	01/11/2018
α-Ethyltryptamine							I	01/11/2018
CP 55,244							CB	01/11/2018
MMAI							AIS	01/11/2018
4-HO-DALT							I	01/11/2018
5-Methyl-MDA							PEA	01/11/2018
4-HO-DBT							I	01/11/2018
4-HO-MPT							I	01/11/2018
4-HO-pyr-T							I	01/11/2018
MDMAI							AIS	01/11/2018
2C-T-21							PEA	01/11/2018
Naloxegol							OPS	01/11/2018
Bromazepam							BZP	01/11/2018
Flunitrazepam							BZP	01/11/2018
Eluxadoline							UNK	16/11/2018
1B-LSD							I	16/11/2018

CB: Cannabinoids, CAT: Cathinones, UNK: Unknown, P&E: Plants & extracts, AIS: Aminoindanes.

Appendix B | Masses corresponding to first, second and third fragments (on the right, at the centre and on the left, respectively). The green colour represents the values to rectify and the red ones are not able to find.

Row Labels	Count of ID
91.0535	1
109.0454	1
121.0658	1
150.0185	1
152.0621	1
162.1267	1
165.0734	1
179.0524	1
181.1014	1
197.1285	1
202.1590	1
203.1064	1
208.1154	1
216.1747	1
243.1853	1
245.0921	1
255.1487	1
264.1747	1
269.0743	1
308.0816	1
309.1809	1
Total	**21**

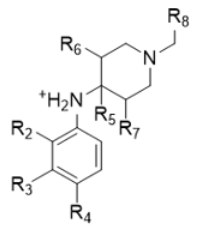
Row Labels	Count of ID
55.0178	1
85.0104	1
88.0758	1
115.0542	3
142.1589	1
147.1034	1
152.0621	1
154.1586	1
165.0883	1
168.1752	1
169.1701	1
175.1113	1
189.1282	1
194.0937	1
224.1281	2
230.0812	1
244.0968	2
272.1070	1
280.0638	1
295.0900	1
312.9159	1
326.1539	1
340.1696	1
Total	**27**

Row Labels	Count of ID
67.0542	1
87.0913	1
101.0898	1
115.0542	1
121.0647	1
128.1069	1
131.0849	1
158.1176	1
161.0818	1
176.0705	1
176.0706	1
189.1258	1
195.0988	1
214.9178	1
221.1073	1
221.1338	1
231.1206	1
236.1281	1
239.1175	1
242.1365	1
298.1546	1
324.1955	1
Total	**22**

Appendix C | Fentanyl analogues used for fragmentation prediction.

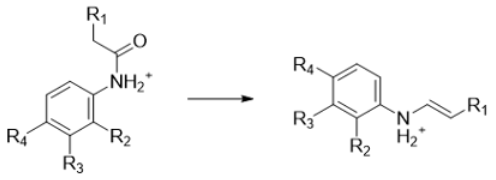
1 2-Fluoro acrylfentanyl
2 2-Fluorobutyryl fentanyl
3 2-Fluoroisobutyryl fentanyl
4 2-Methoxy butyryl fentanyl
5 3-Fluoro methoxyacetyl fentanyl
6 3-Fluorofentanyl
7 3-Fluoroisobutyryl fentanyl
8 3-Methylfentanyl
9 4-Fluoro acrylfentanyl
10 4-Fluorobutyrfentanyl
11 4-Fluorofentanyl
12 4-Methoxy-butyryl fentanyl
13 4-Methyl fentanyl
14 Acetyl fentanyl 4-methylphenethyl analogue
15 Acetyl norfentanyl
16 Acrylfentanyl
17 α-Methyl butyryl fentanyl
18 α-Methyl thiofentanyl
19 α-Methylacetylfentanyl
20 Benzodioxole fentanyl
21 Benzyl carfentanil
22 Benzylfentanyl
23 β-Hydroxythiofentanyl
24 β-Methylfentanyl
25 Butyryl fentanyl
26 Butyryl norfentanyl
27 Carfentanil
28 *cis*-3-Methyl butyryl fentanyl
29 *cis*-3-Methylthiofentanyl
30 *cis*-Mefentanyl
31 Cyclobutyl fentanyl
32 Cyclohexyl fentanyl
33 Cyclopropyl fentanyl
34 Despropionyl 3-fluorofentanyl
35 Despropionyl p-fluoro fentanyl
36 FIBF
37 Furanyl fentanyl
38 Isobutyryl fentanyl
39 Isobutyryl norfentanyl
40 Methoxyacetyl fentanyl
41 Methoxyacetyl norfentanyl
42 *m*-Fluorofentanyl
43 *N*-Methyl norcarfentanil
44 Nor-3-methylfentanyl
45 Norfentanyl
46 Ocfentanil
47 Ohmefentanyl
48 Phenyl fentanyl
49 Sufentanil
50 Thiofentanyl
51 Valeryl fentanyl

Appendix D | Fentanyl analogues: common theoretical masses for fragments a, b, b2, c, c2 and d [143,144].



For fragment a

Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Theoretical mass
10	$-C_2H_5$	-	-	-F	-	-	-	$-C_7H_7$	299.1918
23	$-CH_3$	-	-	-	-	-	-	$-C_5H_5OS$	285.1420
25/38	$-C_2H_5$ **(25)** $-C_2H_6$ **(38)**	-	-	-	-	-	-	$-C_7H_7$	281.2012

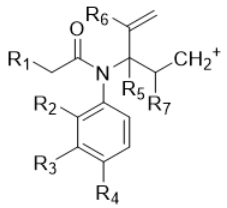


fragment c **fragment c2**

For fragments c and c2

Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Theoretical mass
15	-H	-	-	-	-	-	-	N/A	136.0757 **(c)**
23	$-CH_3$	-	-	-	-	-	-	$-C_5H_5OS$	341.1682 **(c2)**
26/39	$-C_2H_5$ **(26)** $-C_2H_6$ **(39)**	-	-	-	-	-	-	N/A **(26/39)**	164.1070 **(c)**
41	$-OCH_3$	-	-	-	-	-	-	N/A	166.0863 **(c)**
45	$-CH_3$	-	-	-	-	-	-	N/A	150.0913 **(c)**
47	$-CH_3$	-	-	-	-	-	-	$-C_7H_7O$	134.0964 **(c2)**

N/A: not applicable | **26, 39, 41** and **45** contain a hydrogen atom attached to the nitrogen from the piperidine ring.

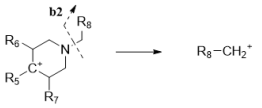


For fragment d

Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Theoretical mass
1	$-CH_2$	-F		-	-	-	-	$-C_7H_7$	232.1132
5	$-OCH_3$	-	-F	-	-	-	-	$-C_7H_7$	250.1238
10	$-C_2H_5$	-	-	-F	-	-	-	$-C_7H_7$	248.1445
12	$-C_2H_5$	-	-	$-OCH_3$	-	-	-	$-C_7H_7$	260.1645
15	-H	-	-	-	-	-	-	N/A	202.1226
33	$-C_2H_4$	-	-	-	-	-	-	$-C_7H_7$	228.1383
38	$-C_2H_6$	-	-	-	-	-	-	$-C_7H_7$	230.1539
51	$-C_3H_7$	-	-	-	-	-	-	$-C_7H_7$	244.1696

N/A: not applicable | **15** contains a hydrogen atom attached to the nitrogen from the piperidine ring.

Appendix D | Fentanyl analogues: common theoretical masses for fragments a, b, b2, c, c2 and d (continued) [143,144].

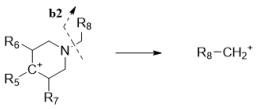


fragment b fragment b2

For fragments b and b2 with a phenethyl group attached to the nitrogen from the piperidine ring

Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Theoretical mass
1	$-CH_2$	-	-	-	-	-	-	$-C_7H_7$	
2	$-C_2H_5$	-F	-	-	-	-	-	$-C_7H_7$	
3	$-C_2H_6$	-F	-	-	-	-	-	$-C_7H_7$	
4	$-C_2H_5$	$-OCH_3$	-	-	-	-	-	$-C_7H_7$	
5	$-OCH_3$	-	-F	-	-	-	-	$-C_7H_7$	
6	$-CH_3$	-	-F	-	-	-	-	$-C_7H_7$	
7	$-C_2H_6$	-	-F	-	-	-	-	$-C_7H_7$	
9	$-CH_2$	-	-	-F	-	-	-	$-C_7H_7$	
11	$-CH_3$	-	-	-F	-	-	-	$-C_7H_7$	
16	$-CH_2$	-	-	-	-	-	-	$-C_7H_7$	188.1434 **(b)**
20	$-C_6H_5$	-	-	-	-	-	-	$-C_7H_7$	105.0699 **(b2)**
31	$-C_6H_5O_2$	-	-	-	-	-	-	$-C_7H_7$	
32	$-C_5H_{10}$	-	-	-	-	-	-	$-C_7H_7$	
33	$-C_2H_4$	-	-	-	-	-	-	$-C_7H_7$	
34	N/A	-	-F	-	-	-	-	-C7H7	
35	N/A	-	-	-F	-	-	-	$-C_7H_7$	
36	$-C_2H_6$	-	-	-F	-	-	-	$-C_7H_7$	
40	$-OCH_3$	-	-	-	-	-	-	$-C_7H_7$	
42	$-CH_3$	-	-F	-	-	-	-	$-C_7H_7$	
46	$-OCH_3$	-F	-	-	-	-	-	$-C_7H_7$	

N/A: not applicable | **34** and **35** do not contain a carbonyl function attached to the nitrogen atom, instead they have a hydrogen



fragment b fragment b2

For fragments b and b2 with different moieties attached to the piperidine ring

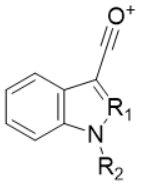
Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Theoretical mass
8	$-CH_3$	-	-F	-	-	-	-	$-C_7H_7$	202.1590 **(b)** 105.0699 **(b2)**
13	$-CH_3$	-	-	-	-	-	-	$-C_8H_9$	202.1590 **(b)** 119.0855 **(b2)**
17	$-C_2H_5$	-	-	-	-	-	-	$-C_7H_7$	202.1590 **(b)** 119.0855 **(b2)**
18	$-CH_3$	-	-	-	-	-	-	$-C_7H_9S$	208.1154 **(b)** 125.0419 **(b2)**
19	-H	-	-	-	-	-	-	$-C_7H_7$	202.1590 **(b)** 119.0855 **(b2)**
22	$-CH_3$	-	-	-	-	-	-	$-C_6H_5$	174.1277 **(b)** 91.0542 **(b2)**
24	$-CH_3$	-	-	-	-	-	-	$-C_8H_9$	202.1590 **(b)** 119.0855 **(b2)**
26/39/ 41/45	$-C_2H_5$ **(26)** $-C_2H_6$ **(39)** $-OCH_3$ **(41)** $-CH_3$ **(45)**	-	-	-	-	-	-	N/A **(26/39/41/45)**	84.0808 **(b)**
27	$-CH_3$	-	-	-	$-C_2H_3O_2$	-	-	$-C_7H_7$	246.1489 **(b)**
28	$-C_2H_5$	-	-	-	-	-	-	$-C_7H_7$	202.1590 **(b)** 105.0699 **(b2)**
29	$-CH_3$	-	-	-	-	$-CH_3$	-	$-C_5H_5S$	208.1154 **(b)** 111.0263 **(b2)**
30	$-CH_3$	-	-	-	-	-	-	$-C_7H_7$	202.1590 **(b)** 105.0699 **(b2)**
47	$-CH_3$	-	-	-	-	$-CH_3$	-	$-C_7H_7O$	218.1539 **(b)**
49	$-CH_3$	-	-	-	$-C_2H_5O$	-	-	$-C_5H_5S$	238.1260 **(b)**
50	$-CH_3$	-	-	-	-	-	-	$-C_5H_5S$	194.0998 **(b)** 111.0263 **(b2)**

N/A: not applicable | **26, 39, 41** and **45** contain a hydrogen atom attached to the nitrogen from the piperidine ring.

Appendix E | Synthetic cannabinoids used for fragmentation prediction.

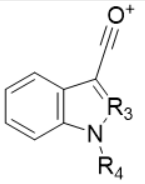
1 1-Naphthoyl indole	**44** A-796260	**87** BB-22 3-carboxyindole metabolite	**130** JWH 200 analog 1	**173** MMB-CHMICA
2 2-Fluoro ADB	**45** A-796260 Degradant	**88** CBL-018	**131** JWH 213	**174** MMB-FUBINACA
3 2-Fluoro AMB	**46** A-834735	**89** CHM-122	**132** JWH 307 3-isomer	**175** MN-18
4 2-Fluoro NNEI	**47** A-834735 Degradant	**90** Cl2201	**133** JWH 387	**176** MO-CHMINACA
5 3-Fluoro ADB	**48** A-836339	**91** CUMYL-PICA	**134** JWH 412	**177** NM-2201
6 3-Fluoro NNEI	**49** AB-005	**92** CUMYL-PINACA	**135** JWH 412 N-(5-hydroxypentyl) metabolite	**178** NNEI
7 4-fluoro ADB	**50** AB-BICA	**93** CUMYL-THPINACA	**136** JWH-007	**179** NNEI 2-indazole isomer
8 4-Fluoro AMB	**51** AB-CHMICA	**94** EAM-2201	**137** JWH-018	**180** NPB-22
9 5-Bromo THJ 018	**52** AB-CHMINACA	**95** EG 018	**138** JWH-019	**181** ORG 28611
10 5-Chloro AKB48	**53** AB-CHMINACA 2-indazole isomer	**96** EG2201	**139** JWH-073	**182** PB-22
11 5-Chloro NNEI	**54** AB-CHMINACA metabolite M1A	**97** EMB-FUBINACA	**140** JWH-081	**183** PB-22 3-carboxyindole metabolite
12 5F-CUMYL-PINACA	**55** AB-CHMINACA metabolite M1B	**98** F2201	**141** JWH-098	**184** PF-03550096
13 5-Fluoro ABICA	**56** AB-CHMINACA metabolite M2	**99** FAB-144	**142** JWH-122	**185** PX 1
14 5-Fluoro AB-PINACA	**57** AB-CHMINACA metabolite M3A	**100** FDU-NNEI	**143** JWH-149	**186** PX 2
15 5-fluoro ADB	**58** AB-CHMINACA metabolite M5A	**101** FDU-PB-22	**144** JWH-180	**187** QUCHIC
16 5-Fluoro ADB metabolite 7	**59** AB-CHMINACA metabolite M6	**102** FUB-144	**145** JWH-198	**188** RCS-4
17 5-Fluoro ADBICA	**60** AB-FUBINACA	**103** FUB-JWH 018	**146** JWH-200	**189** RCS-4-C4-homolog
18 5-Fluoro ADB-PINACA	**61** AB-FUBINACA metabolite 2B	**104** FUB-NPB-22	**147** JWH-203	**190** RCS-8
19 5-Fluoro ADB-PINACA isomer 2	**62** ADB-BINACA	**105** FUB-PB-22	**148** JWH-210	**191** SDB-005
20 5-Fluoro AEB	**63** ADB-CHMICA	**106** JWH 011	**149** JWH-251	**192** SER-601
21 5-Fluoro AMB	**64** ADB-FUBICA	**107** JWH 018 2-hydroxyindole metabolite	**150** JWH-398	**193** STS-135
22 5-Fluoro AMB metabolite 2	**65** ADB-FUBINACA	**108** JWH 018 4-hydroxyindole metabolite	**151** JWH-424	**194** THJ 018
23 5-Fluoro AMB metabolite 5	**66** ADBICA	**109** JWH 018 7-hydroxyindole metabolite	**152** M-144	**195** THJ 2201
24 5-Fluoro AMB metabolite 7	**67** ADB-PINACA	**110** JWH 018 8-quinolinyl carboxamide	**153** MAB-CHMINACA	**196** THJ2201 N-(5-hydroxypentyl) metabolite
25 5-Fluoro BEPIRAPIM	**68** AKB48	**111** JWH 018 adamantyl analog	**154** MAB-CHMINACA metabolite M1	**197** THJ2201 N-pentanoic acid metabolite
26 5-Fluoro CUMYL-P7AICA	**69** AKB48 N-(5-Fluoropentyl) analog	**112** JWH 018 adamantyl carboxamide	**155** MAB-CHMINACA metabolite M10	**198** UR-144
27 5-Fluoro CUMYL-PICA	**70** AM1220	**113** JWH 018 benzimidazole analog	**156** MAB-CHMINACA metabolite M11	**199** UR-144 5-pentanoic acid metabolite
28 5-Fluoro CYPPICA	**71** AM1235	**114** JWH 018 N-(4,5-epoxypentyl) analog	**157** MAB-CHMINACA metabolite M2	**200** UR-144 Degradant
29 5-Fluoro JWH 018 adamantyl analog	**72** AM1241	**115** JWH 018 N-(4-hydroxypentyl) metabolite	**158** MAB-CHMINACA metabolite M3	**201** UR-144 N-(2-Chloropentyl) analog
30 5-Fluoro MDMB-PICA	**73** AM1248	**116** JWH 018 N-pentanoic acid metabolite	**159** MAB-CHMINACA metabolite M7	**202** UR-144 N-(3-Chloropentyl) analog
31 5-Fluoro MDMB-PICA metabolite 7	**74** AM2201	**117** JWH 020	**160** MA-CHMINACA	**203** UR-144 N-(4-Chloropentyl) analog
32 5-fluoro MN-18	**75** AM2201 8-Quinolinyl carboxamide	**118** JWH 022	**161** MAM2201 N-(5-chloropentyl) analog	**204** UR-144 N-(5-Bromopentyl) analog
33 5-Fluoro NNEI	**76** AM2201 Benzimidazole analog	**119** JWH 071	**162** MAM2201 N-pentanoic acid metabolite	**205** UR-144 N-(5-Chloropentyl) analog
34 5-fluoro NPB-22	**77** AM2233	**120** JWH 072	**163** MCHB-1	**206** UR-144 N-(5-Methylhexyl) analog
35 5-fluoro PB-22	**78** AM-630	**121** JWH 073 2-methylnaphthyl analog	**164** MDMB-CHMCZCA	**207** UR-144 N-Heptyl analog
36 5-fluoro PB-22 3-carboxyindole metabolite	**79** AM679	**122** JWH 073 4-hydroxyindole metabolite	**165** MDMB-CHMICA	**208** WIN 54,461
37 5-Fluoro PY-PICA	**80** AM694	**123** JWH 073 4-methylnaphthyl analog	**166** MDMB-CHMINACA	**209** Win 55,212
38 5-Fluoro PY-PINACA	**81** AMB	**124** JWH 073 5-hydroxyindole metabolite	**167** MDMB-FUBINACA	**210** WIN 55,212-2
39 5-Fluoro SDB-005	**82** APP-CHMINACA	**125** JWH 073 N-(3-hydroxybutyl) metabolite	**168** MEP-CHMICA	**211** XLR-11
40 5-Fluoro THJ	**83** APP-FUBINACA	**126** JWH 073 N-(4-hydroxybutyl) metabolite	**169** Methyl-1-(5-fluoropentyl)-1H-indole-3-carboxylate	**212** XLR11 Degradant
41 5-Fluoro-2-ADB-PINACA isomer 2	**84** APP-PICA	**127** JWH 080	**170** Methyl-1-pentyl-1H-indole-3-carboxylate	**213** XLR11 N-(4-hydroxypentyl) metabolite
42 5-Fluoro-3,5-AB-PFUPPYCA	**85** ATHPINACA isomer 2	**128** JWH 081-N-(cyclohexylmethyl) analog	**171** MMB018	**214** XLR11 N-(4-pentenyl) analog
43 5-Fluoropentyl-3-pyridinoylindole	**86** Azidoindolene 1	**129** JWH 122 N-(4-pentenyl) analog	**172** MMB-2201	**215** XLR12

Appendix F | Common theoretical masses for SCs with adamantyl ring containing a carboxyamide or carbonyl functions.



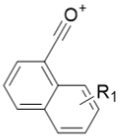
SCs with adamantly ring – carboxyamide function | Fragment b			
Code	**R_1**	**R_2**	**Theoretical mass**
85	-N	$-C_6H_{11}O$	**243.1128** ($C_{14}H_{15}N_2O_2^+$) *core + linker (carbonyl group) + tail*
112	-C	$-C_5H_{11}$	**214.1226** ($C_{14}H_{16}NO^+$) *core + linker (carbonyl group) + tail*
192	N/A	$-C_5H_{11}$	**284.1645** ($C_{18}H_{22}NO_2^+$) *core + linker (carbonyl group) + tail*

N/A: not applicable

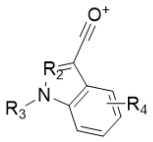


SCs with adamantly ring – carbonyl function | Fragment d			
Code	**R_3**	**R_4**	**Theoretical mass**
73	-C	$-C_7H_{14}N$	**98.0964** ($C_6H_{12}N^+$) | **112.1121** ($C_7H_{14}N^+$) *(tail section)*

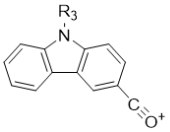
Appendix G | Common theoretical masses for SCs containing a naphthalene ring and a carbonyl group.



SCs containing a naphthalene ring and a carbonyl group | Fragment a					
Code	**R_1**	**R_2**	**R_3**	**R4**	**Theoretical mass**
123	$-CH_3$	-C	$-C_4H_9$	-	169.0648 ($C_{12}H_9O^+$)
127	$-OCH_3$	-C	$-C_4H_9$	-	185.0597 ($C_{12}H_9O_2^+$)
133	-Br	-C	$-C_5H_{11}$	-	232.9597 ($C_{11}H_6BrO^{+)}$
134	-F	-C	$-C_5H_{11}$	-	173.0397 ($C_{11}H_6FO^+$)
144	$-C_3H_7$	-C	$-C_3H_7$	-	197.0961 ($C_{14}H_{13}O^+$)
148	$-C_2H_5$	-C	$-C_5H_{11}$	-	183.0804 ($C_{13}H_{11}O^+$)
150	-Cl	-C	$-C_5H_{11}$	-	189.0102 ($C_{11}H_6ClO^+$)

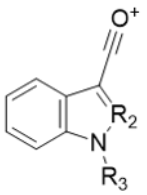


SCs containing a naphthalene ring and a carbonyl group | Fragment b					
Code	**R_1**	**R_2**	**R_3**	**R_4**	**Theoretical mass**
1	-	-C	-H	-	**144.0444** ($C_9H_5NO^+$) *core + linker*
9	-	-N	$-C_5H_{11}$	-	**145.0396** ($C_8H_5N_2O^+$) | **213.1022** ($C_{13}H_{13}N_2O^+$) *core + linker | core + linker + tail*
71	-	-C	$-C_4H_8F$	$-NO_2$	**189.0295** ($C_9H_5N_2O_3^+$) | **277.0983** ($C_{14}H_{14}FN_2O_3^+$) *substituted-core + linker | substituted-core + linker + tail*
90	-Cl	-C	$-C_5H_{10}F$	-	**232.1132** ($C_{14}H_{15}FNO^+$) *core + linker + tail*
103	-	-C	$-C_7H_6F$	-	**109.0448** ($C_7H_6F^+$) | **252.0819** ($C_{16}H_{11}FNO^+$) *tail section | core + linker + tail*
117	-	-C	$-C_7H_{15}$	-	**242.1539** ($C_{16}H_{20}NO^+$) *core + linker + tail*
118	-	-C	$-C_5H_9$	-	**212.1070** ($C_{14}H_{14}NO^+$) *core + linker + tail*
119	-	-C	$-C_2H_5$	-	**172.0757** ($C_{11}H_{10}NO^+$) *core + linker + tail*



SCs containing a naphthalene ring and a carbonyl group | Fragment b – exception					
Code	**R_1**	**R_2**	**R_3**	**R_4**	**Theoretical mass**
95	-	N/A	$-C_5H_{11}$	-	264.0444 ($C_{18}H_{18}NO^+$) core + linker + tail

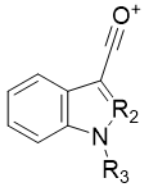
Appendix H | Common theoretical masses for SCs containing a naphthalene ring and a carboxyl group.



SCs containing a naphthalene ring and a carboxyl group | Fragment b

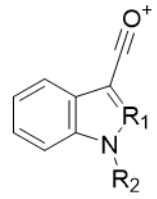
Code	R_1	R_2	R_3	R_4	Theoretical mass
34	-N	-N	$-C_5H_{10}F$	-	**213.1022** ($C_{13}H_{13}N_2O^+$) | **251.1190** ($C_{13}H_{16}FN_2O_2^+$) *core + linker (carbonyl group) + tail | core + linker + tail*
35	-C	-C	$-C_5H_{10}F$	-	**144.0444** ($C_9H_5NO^+$) | **232.1132** ($C_{14}H_{15}FNO^+$) *core + linker (carbonyl group) | core + linker (carbonyl group) + tail*
88	-C	-C	$-C_5H_{11}$	-	**144.0444** ($C_9H_5NO^+$) | **214.1226** ($C_{14}H_{16}NO^+$) *core + linker (carbonyl group) | core + linker (carbonyl group) + tail*
177	-C	-C	$-C_5H_{10}F$	-	**144.0444** ($C_9H_5NO^+$) | **214.1226** ($C_{14}H_{16}NO^+$) *core + linker (carbonyl group) | core + linker (carbonyl group) + tail*
187	-N	-C	$-C_7H_{13}$	-	**97.1012** ($C_7H_6F^+$) | **144.0444** ($C_9H_5NO^+$) | **240.1383** ($C_{16}H_{11}FNO^+$) *tail section | core + linker (carbonyl group) | core + linker (carbonyl group) + tail*
191	-N	-N	$-C_5H_{11}$	-	**145.0396** ($C_8H_5N_2O^+$) | **215.1179** ($C_{13}H_{15}N_2O^+$) | **233.1285** ($C_{13}H_{17}N_2O_2^+$) *core + linker (carbonyl group) | core + linker (carbonyl group) + tail | core + linker + tail*

Appendix I | Common theoretical masses for SCs containing a naphthalene ring and a carboxyamide



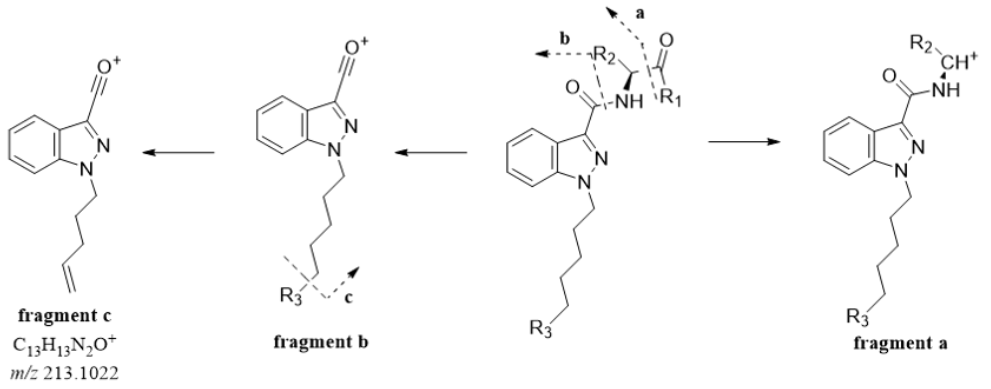
SCs containing a naphthalene ring and a carboxyamide group | Fragment b					
Code	**R_1**	**R_2**	**R_3**	**R_4**	**Theoretical mass**
32	-C	-N	$-C_5H_{10}F$	-	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{16}FN_2O_2^+$) core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail
40	-N	-N	$-C_5H_{10}F$	-	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail
110	-N	-C	$-C_5H_{11}$	-	**144.0444** ($C_9H_5NO^+$) | **214.1226** ($C_{14}H_{16}NO^+$) core + linker (carbonyl group) | core + linker (carbonyl group) + tail
175	-C	-N	$-C_5H_{11}$	-	**145.0396** ($C_8H_5N_2O^+$) | **215.1179** ($C_{13}H_{15}N_2O^+$) core + linker (carbonyl group) | core + linker (carbonyl group) + tail
178	-C	-C	$-C_5H_{11}$	-	**144.0444** ($C_9H_5NO^+$) | **214.1226** ($C_{14}H_{16}NO^+$) core + linker (carbonyl group) | core + linker (carbonyl group) + tail
179	-C	-N	$-C_5H_{11}$	-	**145.0396** ($C_8H_5N_2O^+$) core + linker (carbonyl group)

Appendix J | Common theoretical masses for SCs with TMCP moiety: fragment c.



SCs with TMCP moiety | Fragment c			
Code	**R_1**	**R_2**	**Theoretical mass (linker + core + tail)**
44	-	$-C_6H_{12}NO$	257.1285
46	-	$-C_6H_{11}O$	242.1176
49	-	$-C_7H_{14}N$	242.1414
99/211	-N **(99)**	$-C_5H_{10}F$	233.1085 **(99)** | 232.1132 **(211)**
102	-	$-C_7H_6F$	252.0819
198	-	$-C_5H_{11}$	214.1226
199	-	$-C_5H_9O_2$	244.0968
201	-	$-C_5H_{10}Cl$	248.0837
204	-	$-C_5H_{10}Br$	292.0332
207	-	$-C_7H_{15}$	242.1539
213	-	$-C_5H_{10}FO$	248.1081
214	-	$-C_5H_{10}$	212.1070
215	-	$-C_4H_6F_3$	254.0787

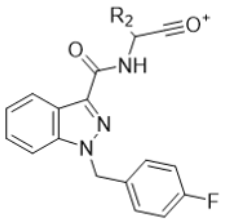
Appendix K | Common theoretical masses for PINACA-SCs.



PINACA-SCs				
Code	**R_1**	**R_2**	**R_3**	**Theoretical mass**
12	N/A	-C_8H_{11}	-F	**91.0542** ($C_7H_7^+$) **119.0855** ($C_9H_{11}^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) *linked group | core + linker (carbonyl group) + tail*
14	-NH_2	-C_3H_7	-F	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) | **332.1769** ($C_{18}H_{23}FN_3O_2^+$) *core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail | core + linker + tail + linked group without the NH_2 group*
18	-NH_2	-C_4H_9	-F	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) | **346.1925** ($C_{19}H_{25}FN_3O_2^+$) *core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail | core + linker + tail + linked group without the NH_2 group*
19	-NH_2	-C_4H_9	-F	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) | **346.1925** ($C_{19}H_{25}FN_3O_2^+$) *core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail | core + linker + tail + linked group without the NH_2 group*
41	-NH_2	-C_4H_9	-F	**213.1022** ($C_{13}H_{13}N_2O^+$) | **233.1085** ($C_{13}H_{14}FN_2O^+$) | **346.1925** ($C_{19}H_{25}FN_3O_2^+$) *core + linker (carbonyl group) + tail | core + linker (carbonyl group) + tail | core + linker + tail + linked group without the NH_2 group*
67	-NH_2	-C_4H_9	-	**215.1179** ($C_{13}H_{15}N_2O^+$) | **328.2020** ($C_{19}H_{26}N_3O_2^+$) *core + linker (carbonyl group) + tail | core + linker + tail + linked group without the NH_2 group*
92	N/A	-C_8H_{11}	-	**91.0542** ($C_7H_7^+$) **119.0855** ($C_9H_{11}^+$) | **215.1179** ($C_{13}H_{15}N_2O^+$) *linked group | core + linker (carbonyl group) + tail*

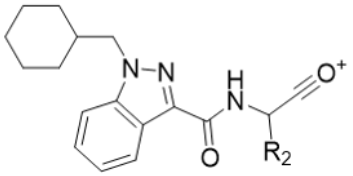
N/A: not applicable. **12 and 92** only comprise the substituents R_2 attached to the carboxamide linker.

Appendix L | Common theoretical masses for FUBINACA-SCs.



FUBINACA-SCs | Fragment a			
Code	**R_1**	**R_2**	**Theoretical mass**
65	$-NH_2$	$-C_3H_7$	**366.1612** ($C_{21}H_{21}FN_3O_2^+$) *core + linker + tail + linked group without the -NH_2 group*
83	$-NH_2$	$-C_7H_7$	**400.1456** ($C_{24}H_{19}FN_3O_2^+$) *core + linker + tail + linked group without the -NH_2* group
97	$-OCH_3$	-	**352.1456** ($C_{20}H_{19}FN_3O_2^+$) *core + linker + tail + linked group without the ester moiety*
167	$-OCH_3$	$-C_3H_7$	**338.1663** ($C_{20}H_{21}FN_3O_2^+$) *core + linker + tail + linked group + without the ester moiety*
174	$-OCH_3$	$-C_3H_7$	**354.1507** ($C_{19}H_{19}FN_3O^+$) *core + linker + tail + linked group without the ester moiety*

Appendix M | Common theoretical masses for CHMINACA-SCs.



CHMINACA-SCs | Fragment a			
Code	**R_1**	**R_2**	**Theoretical mass**
52	$-NH_2$	$-C_3H_7$	**340.2020** ($C_{20}H_{26}N_3O_2^+$) *core + linker + tail + linked group without the -NH_2 group*
53	$-NH_2$	$-C_3H_7$	**340.2020** ($C_{20}H_{26}N_3O_2^+$) *core + linker + tail + linked group without the -NH_2 group*
56	-OH	$-C_3H_7$	**312.2070** ($C_{19}H_{26}N_3O^+$) *core + linker + tail + linked group without the -COOH group*
59	-OH	$-CH_2NO$	**342.1812** ($C_{19}H_{24}N_3O_3^+$) *core + linker + tail + linked group without the amide moiety*
82	$-NH_2$	$-C_7H_7$	**388.2020** ($C_{24}H_{26}N_3O_2^+$) *core + linker + tail + linked group without the -NH_2 group*
153	$-NH_2$	-C3H7	**354.2227** ($C_{21}H_{28}N_3O_2^+$) *core + linker + tail + linked group without the -NH_2 group*
154	$-NH_2$	-C3H7	**370.2125** ($C_{21}H_{28}N_3O_3^+$) *core + linker + tail + linked group without the -NH_2 group*
155	N/A	N/A	**216.1131** (C12H14N3O+) *core + linker + linked group (i.e.-C_4H_8)*
160	$-OCH_3$	$-C_3H_7$	**312.2070** ($C_{19}H_{26}N_3O^+$) *core + linker + tail + linked group without the ester moiety*
166	$-OCH_3$	$-C_4H_9$	**326.2227** ($C_{20}H_{28}N_3O^+$) *core + linker + tail + linked group without the ester moiety*
176	N/A	N/A	***313.1911*** (*$C_{19}H_{25}N_2O_2^+$*) *core + linker + tail + linked group without the ester moiety*

N/A: not applicable. **154** contains a –OH group in the cyclohexylmethyl group, **155** contains a $-C_6H_9O_2$ attached to the carboxyamide linker and **176** comprises a different linker (i.e. a carboxyl function).

Appendix N | List of 36 unknown samples with their respective elemental compositions, precursor ions, fragments and a tentative identification of the substance present in the samples.

Sample	RT (min)	Elemental composition	Accurate mass		Mass error (mDa)	Tentative identification
			Precursor ion $(M+H)^+$	Product ion		
1	3.36	$C_{21}H_{17}NO_2$	208.1332		0.0	Mexedrone
		C_8H_7O		119.0490	-0.1	
		$C_{11}H_{12}N$		158.0964	0.0	
		$C_{11}H_{14}NO$		176.1069	-0.1	
2	5.48	$C_{21}H_{27}N_3O$	338.2224		-0.3	ETH-LAD
		$C_{14}H_{12}N_2$		208.0988	-0.7	
		$C_{16}H_{17}N_2$		237.1380	-0.6	
		$C_{19}H_{23}N_3O$		309.1837	0.1	
3	5.15	$C_{22}H_{27}FN_2O_2$	371.2137		0.8	3-Fluro methoxy acetylfentanyl 4-Fluoro acetylfentanyl Ocfentanil
		C_8H_9		105.0701	0.2	
		$C_{13}H_{18}N$		188.1441	0.7	
4	5.28	$C_{20}H_{25}N_3O$	220.1696		0.4	*N*-ethylhexedrone
		C_7H_7		91.0541	-0.1	
		$C_{10}H_{12}N$		146.0963	-0.1	
		$C_{14}H_{20}N$		202.1593	0.3	
5	6.54	$C_{24}H_{26}N_2O_2$	375.2065		-0.2	Furanyl fentanyl
		C_8H_9		105.0697	-0.2	
		$C_{10}H_{12}N$		146.0961	-0.3	
		$C_{13}H_{18}N$		188.1433	-0.1	
6	11.54	$C_{21}H_{29}FN_2O_3$	377.2232		-0.3	5F-MDMB-PICA
		C_9H_6NO		144.0442	-0.2	
		$C_{14}H_{15}FNO$		232.1129	-0.3	
7	8.73	$C_{16}H_{13}ClN_4S$	329.0620		-0.2	Metizolam
		$C_{14}H_{12}ClN_2S$		275.0401	-0.3	
		$C_{14}H_9ClN_4S$		300.0227	-0.4	
8	11.68	$C_{20}H_{28}FN_3O_3$	378.2185		-0.2	2-Fluoro ADB 3-Fluoro ADB 4-Fluoro ADB 5-Fluoro ADB 5F-MDMB-P4AICA 5F-MDMB-P7AICA
		$C_8H_5N_2O$		145.0394	-0.3	
		$C_{20}H_{28}FN_3O_3$		233.1083	-0.2	
		$C_{13}H_{14}FN_2O$		318.1973	-0.3	
9	8.20	$C_{17}H_{12}ClFN_4$	327.0811		0.4	Flualprazolam
		$C_{17}H_{12}ClFN_4$		292.1121	0.2	
		$C_{17}H_{12}ClFN_4$		299.0624	0.4	
10	8.44	$C_{15}H_{10}BrClN_2O_2$	364.9690		0.3	3-Hydroxyphenazepam
		$C_{13}H_{10}BrN_2$		273.0024	0.2	
		$C_{14}H_9BrClN_2$		318.9633	0.1	
		$C_{15}H_9BrClN_2O$		346.9586	0.5	
11	4.17	$C_{17}H_{25}NO$	260.2010		0.1	3-HO-PCP
		$C_5H_{12}N$		86.0967	0.3	
		C_7H_7O		107.0495	0.4	
		$C_{12}H_{15}O$		175.1119	0.2	
12	6.85	$C_{17}H_{25}NO$	260.2015		0.6	MPHP
		C_8H_9		105.0704	0.5	
		$C_9H_{18}N$		140.1438	0.4	
		$C_{13}H_{17}O$		189.1278	0.4	
13	3.30	$C_{11}H_{16}FN$	182.1345		0.6	2-FEA 3-FEA
		C_7H_6F		109.0453	0.5	
		$C_9H_{10}F$		137.0764	0.3	
14	3.07	$C_{10}H_{12}ClNO$	198.0685		0.5	3-CMC 4-CMC
		$C_{10}H_{11}N$		145.0887	0.1	
		$C_{10}H_{11}ClN$		180.0578	0.4	

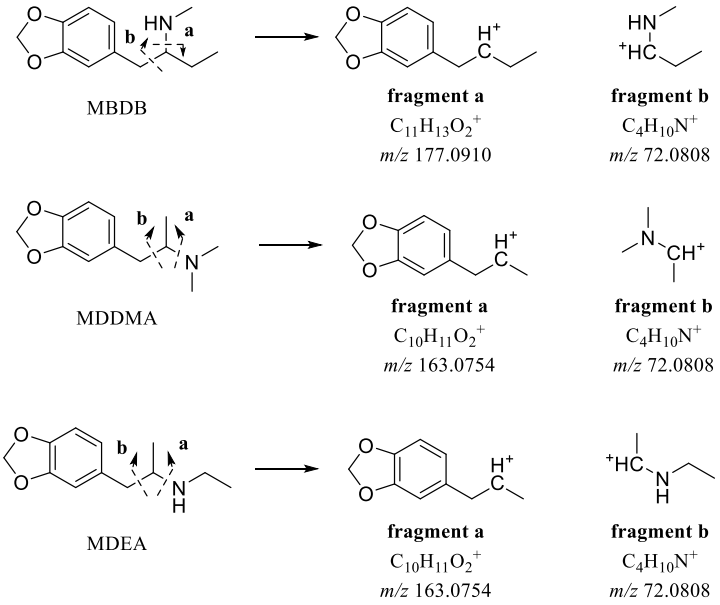
Appendix N | List of 36 unknown samples with their respective elemental compositions, precursor ions, fragments and a tentative identification of the substance present in the samples (continued).

Sample	RT (min)	Elemental composition	Accurate mass		Mass error (mDa)	Tentative identification
			Precursor ion $(M+H)^+$	Product ion		
15	5.73	$C_{161}H_{19}N$	226.1593		0.3	Ephenidine
		C_8H_7O		103.0542	0.0	
		$C_{11}H_{12}N$		181.1014	0.2	
		$C_{11}H_{14}NO$		176.1069	-0.1	
16	6.15	$C_{16}H_{22}Cl_2N_2O$	329.1182		0.0	U-47700
		$C_7H_3Cl_2O$		172.9556	0.1	
		$C_8H_8Cl_2NO$		203.9985	0.8	
		$C_{16}H_{17}N_2$		284.0612	0.9	
17	|5.40	$C_{20}H_{25}N_3O$	324.2065		-0.5	Iso-LSD
		$C_{14}H_{10}NO$		208.0761	0.4	
		$C_{15}H_{15}N_2$		223.1224	-0.6	
		$C_{18}H_{21}N_2O$		281.1636	-1.2	
18	11.68	$C_{20}H_{28}FN_3O_3$	378.2185		-0.2	2-Fluoro ADB 3-Fluoro ADB 4-Fluoro ADB 5-Fluoro ADB 5F-MDMB-P4AICA 5F-MDMB-P7AICA
		$C_8H_5N_2O$		145.0394	-0.3	
		$C_{20}H_{28}FN_3O_3$		233.1083	-0.2	
		$C_{13}H_{14}FN_2O$		318.1973	-0.3	
19	8.20	$C_{17}H_{12}ClFN_4$	327.0811		0.4	Flualprazolam
		$C_{17}H_{12}ClFN_4$		292.1121	0.2	
		$C_{17}H_{12}ClFN_4$		299.0624	0.4	
20	6.93	$C_{17}H_{12}FN_5O_2$	338.1049		0.2	Flunitrazolam
		$C_{16}H_{11}FN_3$		264.0936	0.5	
		$C_{17}H_{13}FN_4$		292.1117	-0.2	
		$C_{16}H_{11}FN_4O_2$		300.0859	-0.1	
21	5.20	$C_{20}H_{25}N_3O$	220.1696		0.4	*N*-ethylhexedrone
		C_7H_7		91.0541	-0.1	
		$C_{10}H_{12}N$		146.0963	-0.1	
		$C_{14}H_{20}N$		202.1593	0.3	
22	3.05	$C_{10}H_{12}ClNO$	198.0685		0.5	3-CMC 4-CMC
		$C_{10}H_{11}N$		145.0887	0.1	
		$C_{10}H_{11}ClN$		180.0578	0.4	
23	5.41	$C_{16}H_{23}NO$	246.1860		0.8	α-PiHP
		C_7H_7		91.0547	0.5	
		C_7H_5O		105.0340	0.5	
		$C_9H_{18}N$		140.1438	0.4	
24	8.15	$C_{17}H_{12}ClFN_4$	327.0811		0.4	Flualprazolam
		$C_{17}H_{12}ClFN_4$		292.1121	0.2	
		$C_{17}H_{12}ClFN_4$		299.0624	0.4	
25	3.29	$C_{11}H_{16}FN$	182.1345		0.6	2-FEA 3-FEA
		C_7H_6F		109.0453	0.5	
		$C_9H_{10}F$		137.0764	0.3	

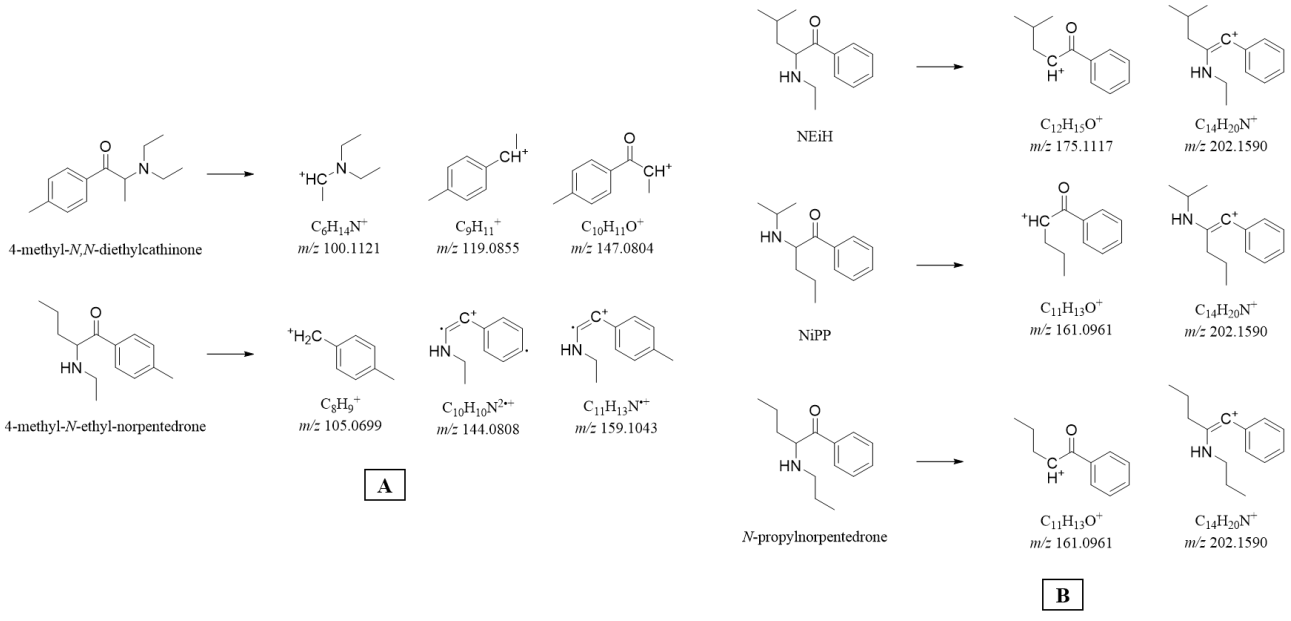
Appendix N | List of 36 unknown samples with their respective elemental compositions, precursor ions, fragments and a tentative identification of the substance present in the samples (continued).

Sample	RT (min)	Elemental composition	Accurate mass		Mass error (mDa)	Tentative identification
			Precursor ion $(M+H)^+$	Product ion		
26	2.48	$C_{11}H_{15}NO_2$	194.1179		0.4	MDMA
		C_7H_5O		105.0339	0.4	
		$C_8H_7O_2$		135.0445	0.4	
		$C_{10}H_{11}O_2$		163.0757	0.3	
27	6.93	$C_{17}H_{12}FN_5O_2$	338.1049		0.2	Flunitrazolam
		$C_{16}H_{11}FN_3$		264.0936	0.5	
		$C_{17}H_{13}FN_4$		292.1117	-0.2	
		$C_{16}H_{11}FN_4O_2$		300.0859	-0.1	
28	6.93	$C_{17}H_{12}FN_5O_2$	338.1049		0.2	Flunitrazolam
		$C_{16}H_{11}FN_3$		264.0936	0.5	
		$C_{17}H_{13}FN_4$		292.1117	-0.2	
		$C_{16}H_{11}FN_4O_2$		300.0859	-0.1	
		$C_{16}H_{11}FN_3$		264.0936	0.5	
29	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
30	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
31	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
32	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
33	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
34	4.02	$C_{21}H_{23}NO_5$	370.1650			Heroin
		$C_{14}H_{11}O_2$		211.0753	-0.1	
		$C_{17}H_{18}NO_2$		268.1331	-0.1	
		$C_{19}H_{22}NO_4$		328.1541	-0.2	
35	4.17	$C_{17}H_{25}NO$	260.2010		-0.1	3-HO-PCP
		$C_5H_{12}N$		86.0967	0.3	
		C_7H_7O		107.0495	0.4	
		$C_{12}H_{15}O$		175.1119	0.2	
36	3.07	$C_{10}H_{12}ClNO$	198.0685		0.5	3-CMC 4-CMC
		$C_{10}H_{11}N$		145.0887	0.1	
		$C_{10}H_{11}ClN$		180.0578	0.4	

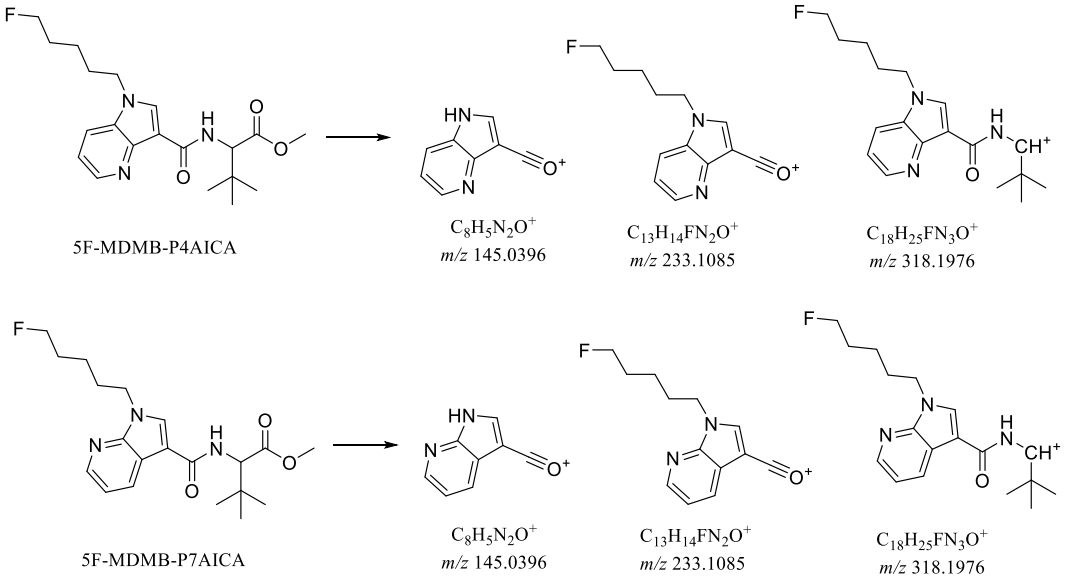
Appendix O | Sample 1: theoretical fragments for MBDB, MDDMA and MDEA [110].



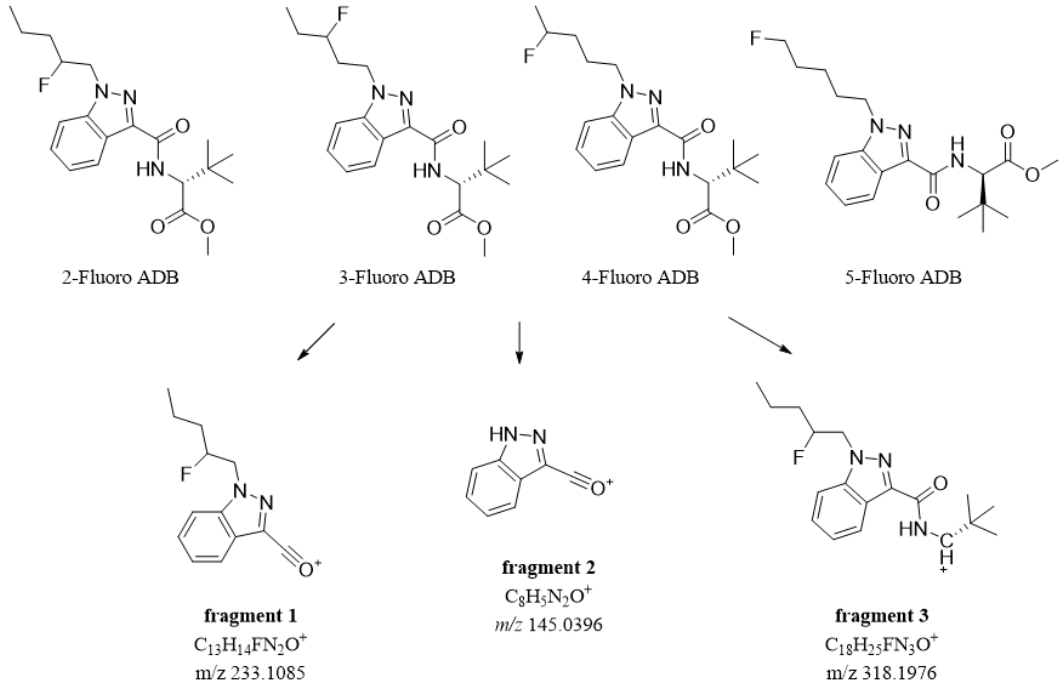
Appendix P | Sample 4: product ions available on HighResNPS (A) and theoretical fragments for NEiH, NiPP and *N*-propylnorpentedrone (B) [151,153].



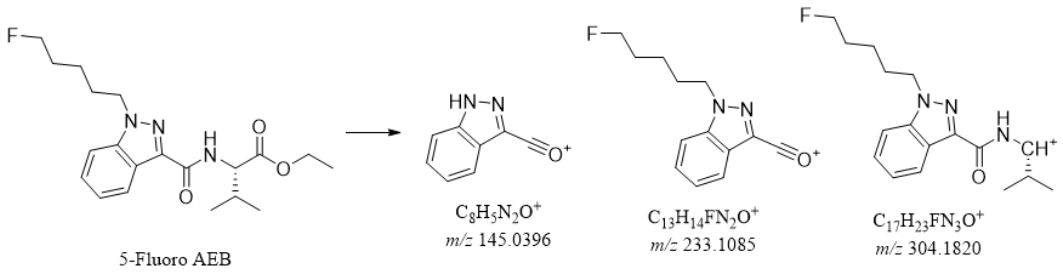
Appendix Q | Sample 8: theoretical fragments for 5F-MDMB-P4AICA [44].



Appendix R | Sample 8: structure of product ions (A) and information on fragmentation available on HighResNPS (B) for 2-fluoro ADB, 3-fluoro ADB, 4.fluoro ADB, 5-fluoro ADB and 5-fluoro AEB.



Note: the fluorine atom can be at position 2, 3, 4 and 5 at the alkyl chain (tail section). The example illustrates the halogen at the position 2, however it is important to highlight that the product ions 1 and 3 will be identical no matter its location.



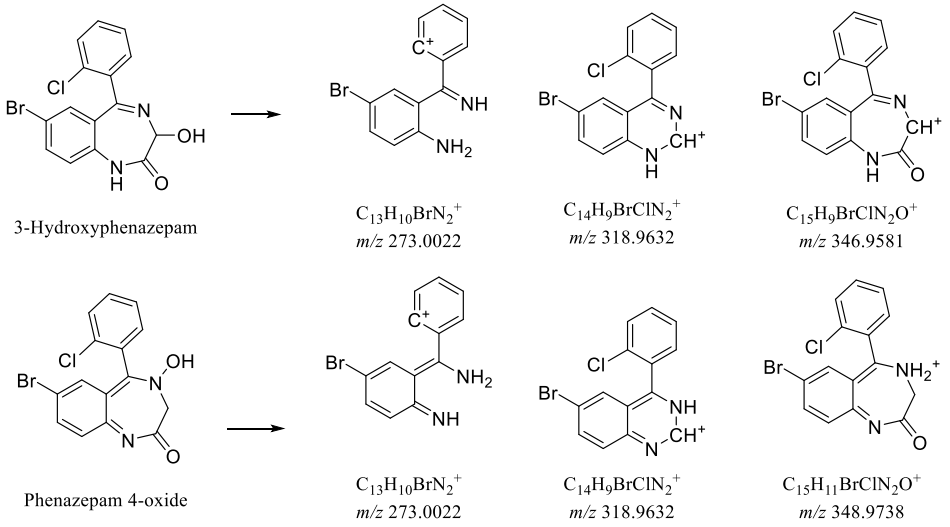
A

Appendix R | Sample 8: structure of product ions (A) and information on fragmentation available on HighResNPS (B) for 2-fluoro ADB, 3-fluoro ADB, 4.fluoro ADB, 5-fluoro ADB and 5-fluoro AEB (continued).

Compound	Laboratory	HighResNPS database			mzcloud™ | CE= 40 eV
		F1MF *F1mass*	F2MF *F2mass*	F3MF *F3mass*	Fragments
2-Fluoro ADB	N/A	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_{18}H_{25}FN_3O$** ***318.1976***	**$C_8H_5N_2O$** **145.0396**	
3-Fluoro ADB	N/A	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_{18}H_{25}FN_3O$** ***318.1976***	**$C_8H_5N_2O$** **145.0396**	
4-Fluoro ADB	N/A	**$C_{13}H_{14}FN_2O$** ***233.1085***	$C_{13}H_{13}N_2O$ *213.1022*	C_5H_9 69.0699	
5-Fluoro ADB	Australia	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_{18}H_{25}FN_3O$** ***318.1976***	**$C_{13}H_{13}N_2O$** ***213.1022***	69.0699 **145.0396** 213.1022 **233.1085** **318.1976**
	Denmark	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_{18}H_{25}FN_3O$** ***318.1976***	**$C_8H_5N_2O$** **145.0396**	
	Denmark	**$C_{13}H_{14}FN_2O$** ***233.1085***	*$C_{13}H_{13}N_2O$* *213.1022*	*C_5H_9* *69.0699*	
	Germany	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_8H_5N_2O$** **145.0396**	**$C_{18}H_{25}FN_3O$** ***318.1976***	
	Greece	**$C_{13}H_{14}FN_2O$** ***233.1085***	**$C_{18}H_{25}FN_3O$** ***318.1976***	$C_{13}H_{13}N_2O$ *213.1022*	
5-Fluoro AEB	N/A	**$C_{13}H_{14}FN_2O$** ***233.1085***	$C_{13}H_{13}N_2O$ *213.1022*	C_5H_9 69.0699	69.0699 **145.0396** 213.1022 **233.1085** 304.1820
5F-MDMB-P4AICA	N/A	-	-	-	-
5F-MDMB-P7AICA	N/A	-	-	-	-

B

Appendix S | Sample 10: theoretical fragments for 3-hydroxyphenazepam and phenazepam 4-oxide [156].



Appendix T | Samples 11 and 12: Theoretical fragments for MPHP and PV8 [157].